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Some Modern Contributions to the Enzyme Theory of Pharmacology*

By MELVIN W. GREEN

When it was demonstrated that sulfonamide activity can be explained, at least in part, by competition with *p*-aminobenzoic acid for some enzyme-controlled metabolic system, increased interest was displayed in the ability of drugs to compete with essential metabolites. Although this conception of drug activity started with anti-infective agents, it soon spread to the vitamin field where it was shown that the administration of many analogues of vitamins can produce vitamin deficiencies. A probable explanation of salicylate poisoning in terms of vitamin K competition is also offered. This concept of drug action, reviewed here, offers a new point of departure in chemotherapy and a means of explaining the mechanism of action of certain drugs.

FOR MANY decades interest has been displayed in the mechanism by which drugs act in the human body and the search for the answer to this question has brought about a distinct professional group, the pharmacologists, dedicated to its search. After the discovery of enzymes in the 19th century, it soon became evident that at least one possible mechanism was the modification of enzyme-controlled metabolic processes. Clarke and many of the early workers showed some evidence, largely on theoretical grounds, that such a concept was tenable. Recent developments in enzyme technology have brought attention dramatically to such a theory.

The enzyme concept of pharmacology now rests upon such a firm foundation that this paper can hope to review only a few of the most salient and recent contributions. The reviewer is hoping to interest pharmacologists in channeling research in this direction rather than attempting to write a comprehensive review. It is believed by the reviewer that this conception of the mechanism of drug action is one of the most stimulating of all theories propounded to date and will form the basis for much of the therapy of the future.

Most of the present interest in the enzyme theory of drug action dates from the dis-

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covery of Loewi (1) in 1921 that parasympathetic stimulation results in the liberation of acetylcholine which, when it comes in contact with the cells in question, produces the characteristic physiological response. It had been known for some time that physostigmine was parasympathomimetic in nature and shortly after Loewi's discovery, it was demonstrated that this action is in reality due to an inhibition of cholinesterase, the enzyme responsible for the physiological hydrolysis of acetylcholine. It has been demonstrated also that drugs not administered specifically for their parasympathetic effects often elicit side-actions through such an enzyme mediation. For example, the Bernheims, Wright (2, 28), and others have shown that morphine inhibits cholinesterase, a factor that probably accounts for the cardiac slowing and the respiratory depression of morphine. In fact it is not altogether unlikely that this enzyme inhibition may be responsible for the central stimulation. This parasympathetic phase of pharmacology has been discussed and reviewed adequately elsewhere (3, 4, 29).

For some time it has been known that when sympathetic nerves are stimulated, sympathin, a substance many people believe to be either epinephrine or a structural analog, is liberated and affects the receptors in a manner analogous to acetylcholine. There is considerable evidence to show that cocaine, ephedrine, and certain sympathomimetic agents act by the inhibition of the enzymes destroying the sympathin and/or epinephrine. Since it is not clear whether epinephrine is destroyed by catechol oxidase (5), amine oxidase (6), or by conjugating with sulfate (7), an analysis of the physiological significance of this mechanism is difficult at this time. The pharmacological action of the sympathomimetic amines was most recently reviewed at the American Chemical Society Symposium in 1944 (8).

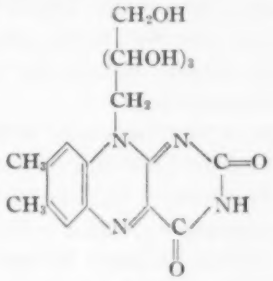
The spectacular advent of the sulfonamides caused considerable search for an enzymic mechanism to explain their action. Early attempts to tie the action of the sulfonamides to carbonic anhydrase and certain other enzymes were more or less abortive. The finding of Woods (9) that the action of these drugs could be inhibited by para-amino benzoic acid (PABA) soon brought to light the activity of PABA as an important link in metabolic function within animal tissues as well as bacteria. This finding seems to carry with it the idea that the sulfonamides can compete with PABA for certain vital centers in an enzyme in bacteria (possibly in other tissues too) and, indeed, may be the successful competitor. This competition is due to the structural similarity, but the similarity in action seems to end once the drug has successfully kept the PABA away from the enzyme center, for the sulfonamide is not capable of replacing the PABA in the metabolic events in which the latter participates.

In an analogous manner, McIlwain (10) found that pyridine-3-sulfonic acid could compete with nicotinic acid. Thus the stage was set for the possible production of vitamin deficiency in higher animals by the feeding of structural analogs of the vitamins.

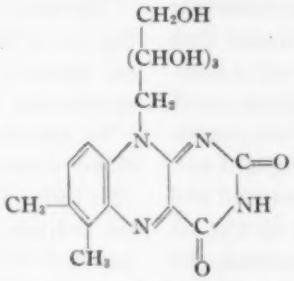
In 1943, Woolley and White (11) produced an apparent thiamine deficiency in mice by feeding a pyridine analogue, 2-methyl-4-amino - 5 - pyrimidylmethyl - (2 - methyl - 3-hydroxyethyl) pyridium bromide. Normally, mice respond differently from rats to thiamine deficiency in that they simply consume less and less food until they die (12), but the feeding of the above compound, commonly called pyrithiamine, was found to produce the thiamine deficiency symptoms without any severe anorexia. On the fourth or fifth day, the animals assumed the characteristic hunched position. This was followed by tremors, convulsions, failure to use the hind legs well and walking around in circles. No animals survived longer than three days after the onset of these symptoms. It was shown that 40 moles of pyrithiamine nullified the action of 1 mole of thiamine.

Emerson and Tishler (13) showed that the feeding of isoriboflavin could produce a riboflavin deficiency. Woolley (14) found that 2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine could compete with riboflavin in certain microorganisms. The

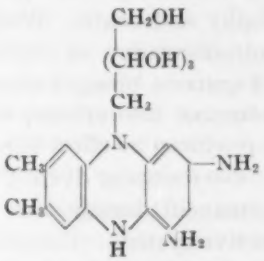
formulas, below, show the similarity of these compounds in structure.



Riboflavin



Isoriboflavin



2,4-Diamino-7,8-dimethyl-10-ribityl-5-10 dihydrophenazine

With but one exception, the microorganisms which were prevented from growing required riboflavin in their media and, conversely, those organisms not affected by the phenazine compound were not stimulated by riboflavin. The dinitro-derivative (used because of its greater stability) produced a mild riboflavin deficiency in mice, but even when given in large doses the deficiency was not fatal. The symptoms of decreased growth, greasy unkempt fur, and hyperirritability were overcome with riboflavin, however.

In 1938, Woolley and co-workers (15) showed that 3-acetylpyridine promptly killed dogs which were nicotinic acid-deficient but had no effect on normal dogs. Mice do not ordinarily respond to nicotinic acid deficiency, yet Woolley (16) found that 3-acetylpyridine produced signs of such deficiency when fed to mice, and these symptoms were prevented by either nicotinic acid or nicotinamide. When mice were given more than 10 mg. of 3-acetylpyridine they exhibited rapid respiration, followed by loss of hind leg control and death within one day. The dose of 2 to 4 mg. showed the best sequence of events, i.e., immediate respiratory augmentation, some hind quarter paralysis in a few hours, and complete paralysis in about two days. Emaciation, and red and inflamed skin soon occurred, and in about four to seven days a fiery red tongue developed. This last symptom is slow to develop in human deficiencies also. Typical of Woolley's data is the finding that 4 mg. of 3-acetylpyridine gave an average survival time of three days, while 4 mg. of the pyridine derivative, along with 2 per cent of nicotinic acid in the diet, gave a survival time of more than fourteen days, and in fact nicotinic acid at the 0.2 per cent level extended the survival about the same degree. Peculiarly, 3-acetylpyridine had no appreciable growth inhibitory properties on *Lactobacillus casei*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Staphlococcus aureus*, and *Lactobacillus arabinosus*. This is difficult to explain since it was shown that these organisms had no appreciable power of destroying the 3-acetylpyridine.

In 1943, Woolley and Krampitz (17) showed that glucoascorbic acid when added at the 5 to 10 per cent level to the diets of mice, could produce scurvy in mice, animals which are not capable of becoming scorbutic on diets totally devoid of vitamin C. Glucoascorbic acid is 2,3-enediol-*d*-glucoheptono-1,4-lactone and bears the same relationship to glucose as ascorbic acid does to xylose. In four or five days the mice developed severe diarrhea, and at about the seventh day severe subcutaneous hemorrhages were seen, especially on the chest and finally on the legs, tail, sides, and gingiva. At autopsy, in two weeks, the rib joints were fiery red. X-rays showed poor bone calcification. In this case the administration of ascorbic acid did not protect and neither did the administration of chlorobutanol, which Longenecker, Fricke, and King (18) had previously shown produced increased vitamin C production *in situ* in rats.

In 1945, Woolley showed that α-tocopherol quinone (19) has biological properties linked both to vitamin E and to vitamin K. The typical sign of vitamin E (tocopherol) deficiency is the death and resorption

of the fetus (rats and mice) during the latter part of the period of gestation. Only a prolonged deficiency can produce muscular dystrophy and death. Woolley found that the administration of 100 mg. of α-tocopherol quinone brought about resorption of the fetus and hemorrhage, while comparable doses produced no effect in nonpregnant animals. No muscular dystrophy occurred and no permanent damage was done to the reproductive system. Tocopherol acetate did not prevent the symptoms from developing. Prompted by the intense bleeding from the vagina, vitamin K was given and was found to prevent both the hemorrhage and resorption. Unlike a similar structural antagonist to vitamin K, 3,3′-methylenebis-(4-hydroxycoumarin), the effect of the α-tocopherol quinone is limited to pregnant mice and the reproductive system, for there is no increase in the prothrombin time. It is also interesting to note that 3,3′-methylenebis-(4-hydroxycoumarin) caused no interruption of pregnancy. More space cannot be devoted here to this interesting observation but it is worth noting that Tishler and Evans (20) have described a compound, naphthotocopherol, which has both vitamin K and vitamin E activity.

An interesting 1944 paper of Woolley (21) was inspired by earlier observations by Goodman and Gilman (22) on some pharmacological properties of benzimidazole. Woolley found that benzimidazole could inhibit the growth of certain microorganisms, but despite its structural similarity to biotin, the latter could not prevent this action. However, he showed that the growth-inhibiting property could be overcome by the addition of adenine and guanine (aminopurines) to the culture medium. The compounds 4-aminobenzimidazole and 4-nitrobenzimidazole, which are structurally more nearly like adenine, were just about as active as the unsubstituted benzimidazole, while 5-aminobenzimidazole was only half as active.

It is significant that in some of these drug-induced deficiencies, the deficiency can be produced only in those organisms capable of growing only in the presence of the requisite vitamin, but just as sulfanilamide is not limited in its actions to organisms which require PABA, benzimidazole is not limited to organisms requiring adenine.

The aminopurines do not alter appreciably the anesthetic activity of benzimidazole, but adenine does reduce slightly the anesthetic time of benzimidazole.

In an attempt to find the cause of sweet clover disease in cattle, Link and co-workers (23) found that dicoumarol [3,3′-methylenebis-(4-hydroxycoumarin)], which could be isolated to the extent of about 60 mg. per Kg. of spoiled clover hay, was the active agent. Of particular interest here is the finding of Link (24) that the feeding of vitamin K could overcome the anticoagulant properties of dicoumarol. Link found that any great change in the structure of dicoumarol brought about a marked change or even loss of activity, but significantly all compounds which were active could, at least theoretically, be degraded into salicylic acid. When salicylic acid itself was fed to rats on a diet low in vitamin K, Link (25) found a hypoprothrombinemia similar to that produced by dicoumarol. It is significant that salicylates do not appear to affect the prothrombin activity of rabbits and dogs, species which are resistant to avitaminosis-K. Rapoport, *et al.* (26) showed that aspirin lowers the prothrombin content of human blood. To further cement this vitamin K–salicylate antagonism is the interesting observation of Shemiakin and Schukina (27) that quinones capable of exhibiting vitamin K activity can be degraded to phthallic acid. Furthermore, after feeding 2-methyl-1,4-naphthoquinone (vitamin K_3) to a dog, phthallic acid was recovered from the urine. When one considers the structural similarity of phthallic acid to salicylic acid, it is not difficult to believe that salicylates (and dicoumarol) can cause hemorrhage from hypoprothrombinemia by competing with phthallic acid, formed during metabolisms of vitamin K, for some enzyme center.

These examples, and others recorded in the literature, show that a positive concept of deficiency disease as well as the historically negative one exists. It remains to show how this concept may be translated into a working pharmacological philosophy.

Researches such as these just discussed serve to point up the fact that profound metabolic changes can be brought about by enzyme inhibitors and structural analogs of the essential metabolites. No doubt the number of such inhibitors is very large. It would be of interest to examine some of the finer effects of some of these substances.

While the production of a vitamin deficiency by positive means is dramatic, the production of a partial and localized deficiency might be of more practical application. The B vitamins, especially, have been linked closely with cellular respiration for well over a decade. The inhibitors of these vitamins, if fed at a lower dosage, might bring about a localized vitamin deficiency, causing hyperactive tissues to operate at a lower energy level. This might be very useful clinically in the treatment of certain diseases of the central nervous system; for example, the feeding of structural analogs of some of the hormones might have important implicatoins, too.

A precedent for such a possibility exists in pharmacology. There are many examples of series of drugs in which small chemical changes sufficiently alter such physical properties as solubility in water or lipoids, surface tension, diffusibility, polarity, dissociability, etc., so that a considerable measure of tissue and organ specificity exists. The sympathomimetic amines are exemplary. The administration of these drugs simulates stimulation of the sympathetic nervous system, i.e., elevation of blood pressure, relaxation of bronchii, inhibition of gastrointestinal motility, and mydriasis. Yet certain of these amines can cause bronchial relaxation with a limited pressor activity; thus, at a given dose level, a measurable degree of tissue specificity exists. In this same manner, no doubt, a study of a series of vitamin inhibitors may disclose compounds of clinical value for their specificity in influencing metabolism. Certainly such recent work points out clearly the need for a more thorough study of the effect of drugs, both old and new, on enzyme-mediated metabolic processes.

This approach has been reviewed recently by Woolley (30), McIlwain (31), and Wagner-Jauregg (32). Since this paper was submitted for publication, a particularly fine review of this subject by Welch (33) appeared to which the reader is referred for a wealth of detail.

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A Revised Cylinder-Plate Assay for Penicillin*

By C. I. BLISS†,‡

A revision is proposed of the cylinder-plate assay for penicillin in which two concentrations of an unknown preparation are compared with two equivalent concentrations of the working standard. A complete assay consists of three four-plate groups, each testing a separately weighed sample of the dry drug. Potency can be estimated from a few simple differences and sums with the aid of two charts, while two other charts provide an estimate of the percentage standard error. The procedure is illustrated with a numerical example. Five requirements for an acceptable assay are proposed.

A CYLINDER-plate assay for penicillin has been tested recently in a collaborative study sponsored by the U. S. Pharmacopœia. On the basis of these findings (3), a revised plate assay is now proposed that is suitable for dry penicillin in bulk. It is simple enough to be handled by the average laboratory technician while giving more reliable results than before. Starting with a few simple differences and their sums, potency in Penicillin Units per mg. can be obtained directly from a simple figure. With the aid of one other figure,[1] the method also provides criteria of its reliability. Instead of this graphic solution, the potency and a test of its reliability may be determined from simple algebraic equations if desired.

EXPERIMENTAL

Laboratory Procedure.—As has been described elsewhere (2), the assay depends upon comparing two concentrations of the Reference Standard with two equivalent concentrations of the sample or unknown. Each plate carries all four concentrations and four similar plates comprise an assay group. A complete assay consists of three or more assay groups, although in many cases it should be possible to check a claimed potency with one or two groups. Larger Petri dishes which will handle six cylinders may be used to assay two unknowns against the same standard. In this case each unknown is compared separately with the standard without other change in technique.

Preparation of Stock Solutions of Standard.—The stock solution of Reference Standard is prepared from 5 to 10 mg. of the Working Standard weighed to the nearest 0.01 mg. A given stock solution of the standard must not be used longer than three days and three different stock solutions of the standard are used in the assay of each unknown. Stock solutions of the unknown are not needed since the test solutions for each unknown sample on a given day are prepared freshly from 5 to 10 mg. of dry penicillin weighed accurately to the nearest 0.01 mg.

Preparation of Test Solutions.—Two test solutions are prepared from the stock solution of the standard. The stronger solution designated as S_H ("standard high") contains the equivalent of 2.7 micrograms of the Working Standard (1 Penicillin Unit) in each cc. The weaker dilution designated as S_L ("standard low") is prepared by adding 2.31 cc. of S_H to 5.00 cc. of buffer solution, or 4.00 cc. of S_H to 8.65 cc. of buffer solution, as may be preferred. The ratio of the test solutions is then 1 to 3.16 which both simplifies the arithmetic in computing the potency and reduces the risk of exceeding the linear part of the dosage-response curve.

Two similar test solutions are prepared from the accurately weighed sample of unknown. It is assumed that preliminary tests have established the potency to within 45% of its final value. On the basis of this preliminary information, the weighed portion of the unknown is dissolved in sufficient buffer solution to make a test solution containing an estimated 1 Penicillin Unit in each cc. designated as U_H ("unknown high"). A weaker dilution designated as U_L ("unknown low") is prepared either by adding 2.31 of U_H to 5.00 cc. of buffer solution or by adding 4.00 cc. of U_H to 8.65 cc. of buffer solution. The assumed potency must not be changed between the assay groups of a complete assay.

Assay Procedure.—Four plates are prepared for each assay group as described in the instructions issued by the Food and Drug Administration (6). One cylinder on each plate is filled with the test solution S_H and the opposite cylinder with the test solution S_L. The remaining two cylinders are filled with the test solutions of the unknown U_H and U_L, respectively. The plates are carefully placed in racks and incubated for sixteen to eighteen hours at 37° C. After incubation the diameter of each circle of inhibition is measured to the nearest 0.2 mm.

The above assay group is repeated on two other days to obtain at least three groups which meet the requirements specified later. Each group tests a different, freshly weighed portion of the unknown penicillin against a different stock solution of the standard.

ESTIMATION OF POTENCY

The potency is estimated from two values T_1 and T_2, determined for each assay group as described in the next paragraph. For a graphic estimate they are used with Charts 1 and 2, which are self-explanatory (Fig. 1). The values T_1 and T_2 from each group are located on Chart 1 and connected by a straight edge to obtain the value M', which is given the same algebraic sign (+ or −) as T_1. M' is determined for each of the three independent assay groups and the three values are summed. Their algebraic sum, $M_1' + M_2' + M_3' = S(M')$, is applied to Chart 2 with the assumed unitage of the

* Received June 4, 1945, from the Dept. of Pharmacology, Yale University, New Haven, Conn.

[1] Large-scale copies of the figures are available on application from the Department of Pharmacology, Yale University, New Haven, Conn.

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‡ The author is indebted to Mr. M. H. Donahey for preparing the nomographs and to Dr. W. T. Salter for advice in writing the manuscript. This study has been aided by grants from the U. S. P. Remington Research Fund, Wm. R. Warner and Company, and Eli Lilly and Company.

unknown, and the potency in units read directly from the middle scale.

Determination of T_1 and T_2.—For each plate the cleared areas from the standard and the unknown are compared at a high concentration and the difference in their diameters, $U_H - S_H$, is noted. A similar comparison is made at the low concentration, giving the difference $U_L - S_L$. These two differences are added to obtain the value $y_1 = U_H - S_H + U_L - S_L$ for each plate. An assay group provides four such values of y_1 and their sum is designated as T_1.

Similarly, the effect of dilution is measured separately for the unknown and for the standard, respectively. The two differences on each plate are then added to obtain $y_2 = U_H - U_L + S_H - S_L$. The sum of the four y_2's from the four plates of an assay group is the value T_2.

In practice both terms are computed with the convenient Work Form A, which is illustrated with the numerical example in a later section. The values of T_1 and T_2 are applied directly to Chart 1 to obtain M' for each assay group. Alternatively, they may be substituted in Equation I for an arithmetic solution of M'.

Arithmetic Estimation of Potency.—Potency can be estimated rapidly and precisely from the two simple equations upon which the charts are based. Many workers will prefer to calculate their answers to the desired precision and then check them with the charts.

The value M', the relative potency in logarithms, is computed separately from T_1 and T_2 for each assay group as

$$M' = \frac{T_1}{2T_2} \qquad \text{(I)}$$

Potency is estimated in Penicillin Units per mg. as the geometric mean of the values of M' from the N' assay groups. Then

$$\text{Units/mg.} = \text{assumed unitage} \times \text{antilog}\left(\frac{S(M')}{N'}\right) \qquad \text{(II)}$$

where the "assumed unitage" in units per mg. is that assumed in preparing the test solutions U_H. Equation II is applicable to assays with any number (N') of assay groups but the corresponding Chart 2 is restricted to those where $N' = 3$.

ESTIMATION OF THE STANDARD ERROR

Two estimates of the standard error for comparing one assayed potency with another can be determined with Charts 3 and 4 (Fig. 2) or their equations. One is determined from the variation within assay groups and the other from the agreement between them. Both are needed to guard against overestimating the precision as will be discussed later. For an acceptable assay, neither estimate should exceed a specified limit. Provisionally, the limits in both cases may be set arbitrarily at 10%.

Determination of the Range.—Both estimates are based upon one or more ranges. A range is defined as the difference between the largest and the smallest values in a series. It is a measure of discrepancy. For determining E_1 the range for y_1, y_2, and y_3 are computed within each assay group. As already described, each y_1 and y_2 is the sum of the two single differences on one plate; y_3 is the difference between these single differences or $y_3 = U_H - U_L - S_H + S_L$. A convenient method of calculating y_3 is given in Work Form A. Within each assay group, the smallest is subtracted from the largest of the four values of y_1, of y_2, and of y_3, respectively, and the three ranges are summed. The total range within each group is designated as R.

For determining E_2, the value R_M is required. This is the difference or range between the two most divergent of the three values of M' which have already been determined.

Graphic Estimation of the Standard Error.—The first percentage standard error is based on the internal consistency of the three assay groups, adjusted for comparison with other whole assays from the agreement observed among collaborators (3). It is determined from $S(R)$, which is the sum of the ranges (R) within the three assay groups, and from $S(T_2)$ which is the sum of their respective T_2's. The values of $S(R)$ and of $S(T_2)$ are applied to Chart 3 and the percent error E_1 read from the middle scale.

This first criterion, however, is not adequate alone, so that a second estimate of the standard error, E_2, is derived from Chart 4. The range in the values of M', R_M, is applied to Chart 4 and the percent error E_2 read directly. Thus the second estimate depends upon the consistency between the three assay groups, each representing an independent set of test solutions.

Arithmetic Estimation of the Standard Error.—As with the potency, the two estimates of the standard error are easy to compute arithmetically from Equations III and IV underlying Charts 3 and 4, respectively. Moreover, with the equations, the number of assay groups is not restricted to three.

The percentage standard error E_1 is based on the internal consistency of the assay groups and is computed from the terms required for Chart 3 as

$$E_1 = 100\left\{\text{antilog}\left(\frac{K_1 S(R)}{S(T_2)}\right)\right\} - 100 \qquad \text{(III)}$$

where $K_1 = 0.241$ for three assay groups. The percentage standard error E_2 from the discrepancy between independent estimates of M' should be of about the same magnitude as E_1. It is given by the equation

$$E_2 = 100\{\text{antilog}\,(K_2 R_M)\} - 100 \qquad \text{(IV)}$$

where $K_2 = 0.341$ for three assay groups.

The number of four-plate groups in an assay may vary from time to time. In calculating E_1 and E_2, the following coefficients are then applicable.

TABLE I

No. of Assay Groups, N'	Total No. of Plates	K_1	K_1'	K_2
1	4	0.418	0.1619	...
2	8	0.295	0.1144	0.627
3	12	0.241	0.0935	0.341
4	16	0.209	0.0810	0.243
5	20	0.187	0.0724	0.192

If E_1 consistently exceeds E_2, the adjustment based upon the agreement observed in the collaborative study is presumably too large and may be omitted. The agreement to be expected from the variation within assay groups alone is given by E_1', which is computed from Equation (III) with the substitution of K_1' for K_1. As the experimental technique is improved, E_2 will agree more nearly with E_1' than with E_1.

NUMERICAL EXAMPLE

In practice it is convenient to use two work forms, A and B (Tables II and III), in computing the various values which have been described. The

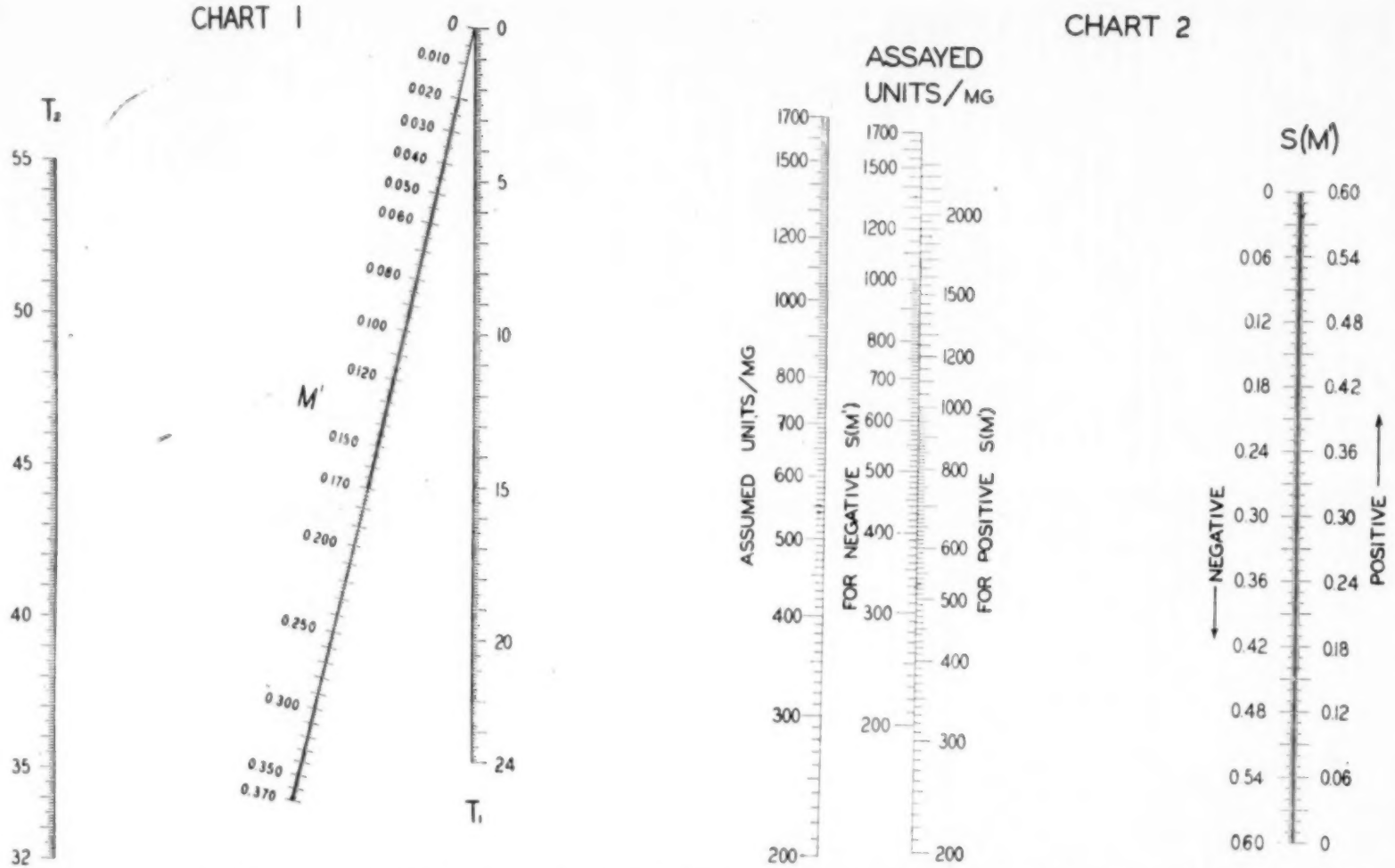

Fig. 1.—Estimation of Potency, based on values T_1 and T_2. (a) Determine M' for each independent assay group with *Chart 1;* give M' the same sign as T_1. (b) Add the three values of M' to obtain $S(M')$. (c) Enter $S(M')$ in *Chart 2* and read off the assayed unitage.

TABLE II.—WORK FORM A

Assay Group No.	Diameter in Mm. for U_H	U_L	S_H	S_L	Initial differences $U_H - S_H = D_1$	$U_L - S_L = D_2$	$U_H - U_L = D_3$	$S_H - S_L = D_4$	Computing values $D_1 + D_2 = y_1$	$D_3 + D_4 = y_2$	$D_1 - D_2 = y_3$
1	24.2	19.2	24.0	18.9	0.2	0.3	5.0	5.1	0.5	10.1	−0.1
	24.4	19.0	23.4	18.7	1.0	0.3	5.4	4.7	1.3	10.1	0.7
	24.2	19.6	24.0	18.9	0.2	0.7	4.6	5.1	0.9	9.7	−0.5
	24.2	19.2	23.6	18.5	0.6	0.7	5.0	5.1	1.3	10.1	−0.1
								Total	4.0	40.0	0
								Range	0.8	0.4	1.2
2	18.8	14.0	18.0	13.5	0.8	0.5	4.8	4.5	1.3	9.3	0.3
	18.8	15.0	18.5	14.5	0.3	0.5	3.8	4.0	0.8	7.8	−0.2
	18.5	13.5	18.5	13.5	0	0	5.0	5.0	0	10.0	0
	19.0	15.0	20.0	14.5	−1.0	0.5	4.0	5.5	−0.5	9.5	−1.5
								Total	1.6	36.6	−1.4
								Range	1.8	2.2	1.8
3	24.0	18.2	24.0	19.0	0	−0.8	5.8	5.0	−0.8	10.8	0.8
	24.2	18.8	25.0	19.0	−0.8	−0.2	5.4	6.0	−1.0	11.4	−0.6
	24.5	18.5	25.0	19.0	−0.5	−0.5	6.0	6.0	−1.0	12.0	0
	24.0	18.0	24.0	19.0	0	−1.0	6.0	5.0	−1.0	11.0	1.0
								Total	−3.8	45.2	1.2
								Range	0.2	1.2	1.6

calculation of potency may be illustrated by a concrete example, embodying the data for three assay groups from three different laboratories in the U. S. P. collaborative study. The assumed potency was 400 units per mg. If the experiments had been carried out in a single laboratory, one would expect closer agreement in slope (y_2) and in the precision with which the diameters were read. The values of y_3 can be computed in two ways, as $y_3 = D_1 - D_2$ and as $y_3 = D_3 - D_4$, which checks the correctness of the initial differences. The total for each y and the total range from each assay group are then assembled in Work Form B (Table III) for the calculation of potency.

TABLE III.—WORK FORM B

Assay Group No.	From values of y: T_1	T_2	T_3	R	M' from Chart 1 or Equation I
1	4.0	40.0	0	2.4	0.050
2	1.6	36.6	−1.4	5.8	0.022
3	−3.8	45.2	1.2	3.0	−0.042
Total		121.8		11.2	0.030
Symbol		$S(T_2)$		$S(R)$	$S(M')$

Entering the assumed unitage (400) and $S(M')$ in Chart 2 (or Equation II), the potency of the unknown is determined as 409 Penicillin Units per mg. The potency has an estimated percentage error from $S(T_2)$ and $S(R)$ by Chart 3 (or Equation III) of $E_1 = 5.2\%$, and from $R_M = 0.050 - (-0.042) = 0.092$ by Chart 4 (or Equation IV) of $E_2 = 7.5\%$.

REQUIREMENTS FOR AN ACCEPTABLE ASSAY

Before acceptance, an assay of three four-plate groups should meet the following five requirements:

1. Test for parallelism. It will be noted from Work Form B that a value designated as T_3 is computed as the sum of the y_3's for each group of four plates. Likewise a value R is obtained indicating the range. Any four-plate assay group in which T_3 exceeds R should be replaced before proceeding with the calculation of potency. For the purpose of this test, T_3 is always taken as a positive number.

2. Small discrepancy between assumed and actual potency. For best results the assumed potency should not differ by more than 45% from the assayed potency. If the summed M' values in Work Form B, $S(M')$, exceeds ±0.48, the determined potency should be regarded only as a pilot experiment.

3. Slope of dosage-response curve. As measured by the summed T_2 values in Work Form B (Table II) this should fall within limiting values of 102.0 and 156.0. These correspond to average slopes (b) between 8.5 and 13.0.

4. The error E_1 must not exceed 10%. Instead of sacrificing the entire assay, it is permissible to replace the four-plate assay group having the largest value of R by a new group.

5. The error E_2 must not exceed 10%. Rather than sacrifice the entire assay, another four-plate group may be added without discarding any data. If E_2 still exceeds 10% with a total of five groups, the most discrepant group may be discarded. If this last device fails, the whole assay should be discarded.

Numerical Example.—These criteria may be illustrated by applying them to the numerical example cited in Work Forms A and B. In this instance the assay is adequate because it meets the following requirements:

1. Parallelism of slopes for standard and unknown: in each assay group T_3 is less than R.

2. Suitable assumed potency: $S(M')$ falls within the limits of −0.48 to 0.48.

3. Mean slope within required limits: $S(T_2)$ is between 102.0 and 156.0.

4 and 5. Errors less than 10%: $E_1 = 5.2\%$ and $E_2 = 7.5\%$.

When the errors were computed from the standard deviations instead of from the ranges, the values corresponding to E_1 and to E_2 were 5.4 and 6.5%, respectively. The latter, of course, are more reliable estimates in that they use all the data instead of just the extreme values, but the values based on the range are close enough approximations for most purposes.

DISCUSSION

The design of the above method is based upon that for randomized groups of four which was computed originally (4) through a factorial analysis of variance. Schild (9) in 1942 and Finney (5) and Sherwood, *et al.* (10) in 1944 showed how the potency could be calculated more directly from the original measurements. This technique was supplemented with a simplified estimate of the standard error (1) for use in the U. S. P. collaborative study (3).

A more recent paper by Knudsen (7) was the first to provide nomographs for estimating potency and its error. Her chart for potency was based upon the same equation as that given by Schild (9) and followed here, but those for error were computed from the range in y_1 and y_2, which she used separately. The present method differs from Knudsen's principally in requiring more than one assay group for the estimation of potency, in providing pooled, adjusted estimates of the experimental error and in eliminating the less reliable values by means of acceptance limits. These acceptance limits must not be confused with statistical control limits. Although the latter would be preferred, especially for E_1 and E_2, they depend upon a continuous check of assay performance in each laboratory.

In most biological assays the use of a reference standard brings the estimates of potency from different laboratories into agreement. In the collaborative study of penicillin, however, the assayed potencies of one unknown varied from 717 ± 17 to 1352 ± 69 units, that of another from 311 ± 8 to 462 ± 8 units, while the other four unknowns showed a similar variability. Even though each range represented twenty laboratories the variation far exceeded expectation. In most laboratories an unknown was assayed with one set of stock

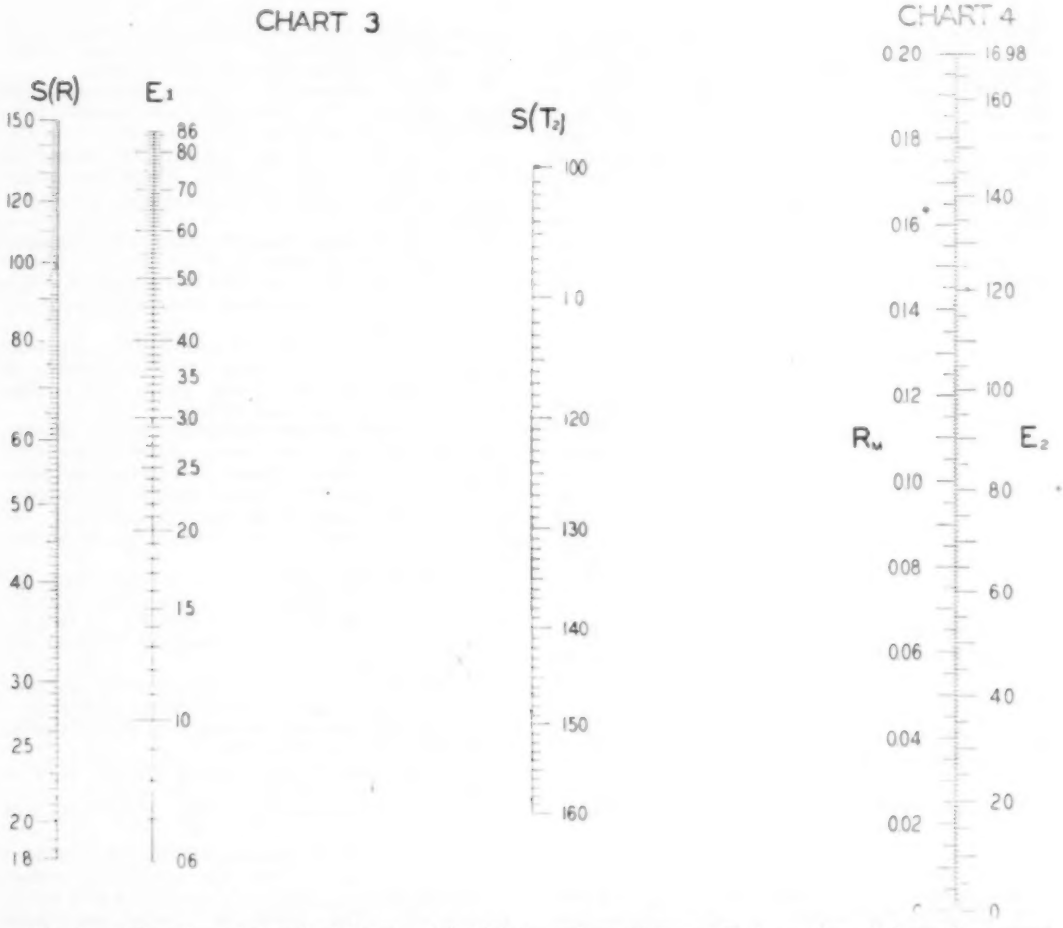

Fig. 2—*Estimation of Standard Error.* (a) Add the values of R and T_2 from three independent assay groups to find $S(R)$ and $S(T_2)$ and enter them in *Chart 3* to find the percentage standard error E_1. (b) Subtract the smallest M' from the largest M' in the set of three assay groups to find R_M and apply it to *Chart 4* to find the percentage standard error E_2.

solutions. Analysis indicates that the discrepancies were due primarily to the difficulty of preparing equipotent solutions from a single lot of dry penicillin. This collaborative result is consistent with reports from several laboratories with large experience in assaying penicillin.

The problem is handled here by requiring that three independent samples be weighed out from a given lot of penicillin and each tested separately against a different stock solution of the standard with a four-plate assay group. These independent estimates, preferably on different days, are then combined into a geometric mean for a final figure. While at least three assay groups are recommended for the original determination of potency, the claimed potency of many samples probably could be checked with one or two such four-plate groups.

An adjustment was required in the standard error (E_1) computed from the internal consistency of the three assay groups. The collaborative study (3) revealed three levels of variation, even after eliminating the four most discrepant laboratories. It may be assumed that further experience would reduce the discrepancy between laboratories at least to that observed among the remaining sixteen collaborators. The first level of variation was that within assay groups, s_M, computed as described before (1). The second was measured from the inconsistency within laboratories as $s_2^2 = 3.06\ s_M^2$. The average inconsistency between laboratories was the third, estimated as $s_3^2 = 2.59\ s_M^2$. The real error in the potencies of the collaborative study was equal to the sum of these three variances, or $s_M^2 + s_2^2 + s_3^2 = 6.65\ s_M^2$. This experience has been drawn upon in adjusting E_1 so that the usual error (s_M) is multiplied by the average discrepancy or by $\sqrt{6.65} = 2.58$. A study of the internal evidence suggests that the second and third levels of variation were due primarily to difficulties in preparing the test solutions of the unknowns and of the standard, respectively. Several collaborators considered this one of the most difficult phases technically with the type of assistance available for routine assays. No doubt other sources of error also contributed to the variability in these collaborative studies, such as trouble with the media or bacteriological difficulties. With more experience better agreement between laboratories would be expected which would reduce the need for the empirical factor of 2.58. It has been introduced in s_M to avoid over-estimating the precision. If preferred, the empirical factor may be omitted by computing E_1' from Equation III with the values for K_1' instead of those for K_1.

In addition to the above adjustment the equation for s_M has been simplified for routine use by estimating the standard deviation from the mean range and by pooling the data from the three assay groups into a single average value. In several laboratories both the standard deviation and the slope varied from day to day beyond their sampling errors. In a number of cases the standard deviation for y_3 exceeded that for y_1 and y_2 (3), not very much but yet significantly. With more experience, these differences would be expected to diminish. Meanwhile, it has seemed better statistical judgment to accept the risk of a small bias in the estimate of $\bar{s}$ and $\bar{b}$ in return for a substantial increase in stability and greater ease of calculation.

Several approximations have simplified the computation of s_M as given in Equation (6) of reference (1). Since T_1 is usually a small fraction of T_2, the ratio in the term $\{1 + T_1^2/T_2^2\}$ has been omitted but its size limited by the allowable discrepancy between the assumed and the assayed potencies. This reduced the equation to $\bar{s}_M = \bar{\lambda}\sqrt{1/N}$, with $\bar{\lambda} = \bar{s}/\bar{b}$ and N = number of plates in all assay groups. The average slope was computed as $\bar{b} = \dfrac{S(T_2)}{N}$. The standard deviation in units of an initial difference was estimated from the mean range as $\bar{s} = \dfrac{S(R)}{2N \times 2.059}$, where 2.059 is the mean range in terms of σ- for samples of four (8). Combining constant terms and multiplying by the factor 2.58 from the preceding paragraph, the standard error of the mean log-potency can be computed as $\bar{s}_M = \dfrac{K_1 S(R)}{S(T_2)}$, K_1 taking the values in the table on page 7.

Although E_1 is increased by a factor to improve its reliability in the comparison of potencies, it involves an approximation. It is desirable to check directly the agreement between the independent determinations of log-potency (M') in the three assay groups. This is provided by E_2 which represents the standard error of the mean where $s_{\bar{M}} = \sqrt{\dfrac{S(M' - \bar{M}')^2}{N(N-1)}}$. Sacrificing efficiency for ease of calculation, the standard deviation may be estimated from the range of the three assay groups as $R_M/1.693$, where 1.693 is the mean range in terms of σ- for samples of three (8). Hence $s_{\bar{M}}$ is equal to $\dfrac{R_M}{1.693\sqrt{3}}$ or $K_2 R_M$. For different numbers of assay

groups, K_2 has the values shown in Table I. Although the proposed assay includes the errors in repeating an assay on different days with independently weighed amounts of drug, it does not test directly the difficulties which one laboratory may have in matching the results of another. Adoption of the present plan, however, should reduce materially the number of such discrepancies as compared with those which might be expected if each laboratory ran only one assay group. The two estimates of error, E_1 and E_2, should be of about the same magnitude and neither should exceed 10 per cent for reasonable assurance that a check assay will confirm the claimed potency.

The other requirements reduce the incidence of aberrant results or simplify the calculation. One rejects assays, at statistical odds of about 1 in 100, in which the dosage-response curves for standard and unknown depart from parallelism. When slopes differed significantly in the collaborative study, duplicate determinations were twice as prone to disagree as when they were parallel. Another limits the difference between the assayed and the assumed potencies of the unknown to 45 per cent. Within these restrictions the contribution to s_M of the variance in the slope is negligible as noted above, so that this component can be omitted in computing the error.

In the current proposal the assay of each unknown is self-contained, even when the plates are large enough to hold six instead of four cylinders and two unknowns are tested against the standard in each group. In laboratories where penicillin is assayed repeatedly a continuous check on procedure will pay dividends. This can be accomplished most effectively with quality control charts of the more important characteristics, such as of the slope, the test for parallelism in slope, the standard deviation or range (used in E_1) and the agreement between independent estimates of the log-potency (E_2). The construction and use of such charts will be discussed elsewhere.

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Notes on the Water Content of Crystalline Caffeine*

By KENNETH L. WATERS† and GEORGE D. BEAL‡,§

A study indicates that under normal storage conditions the stable form of caffeine is the anhydrous salt. The monohydrate, described in the U. S. Pharmacopoeia, apparently does not exist. The water content of hydrated caffeine, which is stable at very high relative humidities, may be represented by the fraction 5/6 mole of water per mole of caffeine. Approximately the same percentage of water is obtained when hydrous caffeine is dried at 80° as when dried at 100°; however, sublimation becomes appreciable at 105°.

THE United States Pharamacopœia (1) first recognized caffeine as an official drug in 1883 when the Sixth Revision described the alkaloid as containing one molecule of water of hydration. The monohydrate was apparently chosen on the basis of Strecker's (2) work in 1861 in which he stated that the alkaloid crystallized with 8.5 per cent water. Later Tassily (3) found that crystalline caffeine when dried at 110° lost 6.93 per cent of its weight; however, he proceeded to establish the formula of the monohydrate on the basis of his nitrogen analysis of the air-dried substance. It is common knowledge that the commercial product seldom, if ever, contains the theoretical 8.49 per cent water; Allen (4) gives 7.0 per cent as the amount usually found. The U. S. P. XII (5) recognizes the instability of the hydrated alkaloid, stating that it is efflorescent in air. U. S. P. XII permits the use of anhydrous caffeine in preparations containing caffeine, but requires

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§ The authors are indebted to Dr. Frank Carruth of the Maywood Chemical Company for suggesting that the one molecule of water shown in the U. S. P. XII formula for caffeine was in error; to Mr. Joseph Rosin, Vice-President of Merck & Company, Inc., for his interest and advice.

that if the preparation be labeled with respect to its caffeine content the statement be in terms of hydrous caffeine.

In order to gain more information on the stability of the hydrate a study was made of the moisture content of crystallized caffeine which had been dried to constant weight at various humidities. The moist crystals "dried" at room temperature, in constant humidity desiccators having a relative humidity approaching 100 per cent, were found to contain approximately 7.0 per cent water. This is far removed from the theoretical 8.49 per cent which would be obtained from the monohydrate.

Samples of moist caffeine dried in desiccators having relative humidities of 60 per cent, or higher, showed an average moisture content of 6.95 per cent, whereas samples dried at relative humidities of 40 per cent or lower became anhydrous. Samples which had become anhydrous did not regain their water content at relative humidities of 60 per cent or 80 per cent, but did regain their constant water content when placed in constant humidity desiccators having a relative humidity of approximately 97 per cent.

As a check on the desiccator experiments two samples containing approximately 7 per cent of water were allowed to stand in open containers in a room having a clean atmosphere with a constant relative humidity of 64 per cent and a constant temperature of 21°. The samples were observed to lose water of hydration slowly; after eight weeks one of the samples was anhydrous although the other sample still contained approximately 30 per cent of its water; twelve weeks were required for this sample to become anhydrous. This behavior accounts for the fact that two previously unopened tins of U. S. P. Caffeine which had a moisture content of 5.25 per cent and 7.01 per cent when packaged two and one half years, and eight years ago, respectively, were found to be anhydrous. These two lots had been on the Mellon Institute shelves for one and six years, respectively.

The results of the moisture determinations, made on samples which had reached a constant weight in desiccators maintained at definite humidities, indicate that the hydrate of caffeine which is stable at high relative humidities contains approximately 7.0 per cent water. The formula for crystalline caffeine may then be more correctly represented with the fractional $^5/_6$ molecule of water rather than with the one molecule of water usually assigned.

A comparison of the loss in weight on drying at 80°, 100°, and 105°, indicates that approximately the same results are obtained at 80° and at 100°; sublimation becomes appreciable at 105°.

EXPERIMENTAL

Constant humidity desiccators were prepared by using saturated solutions of salts in order to approximate relative humidities of 20, 40, 60, 80, and 97%. Potassium acetate, zinc nitrate, sodium bromide, ammonium chloride, and calcium sulfate, respectively, were used for this purpose, and an excess of the solid salt in question was present in each desiccator (6). To approach the two extremes of relative humidity, desiccators were prepared which contained phosphorous pentoxide, and distilled water, respectively.

The two samples which were the subjects of the study were prepared as follows: Sample designated as "A" was prepared according to commercial practices and the freshly separated crystals were rubbed through a coarse sieve and sent to the laboratory; this sample contained approximately 10% water. Sample designated as "B" was prepared in the laboratory by dissolving U. S. P. Caffeine in hot water, allowing it to crystallize overnight, then filtering and pressing dry with a clean cork; this sample contained approximately 20% water.

The procedure followed was to place the moist caffeine in tared dishes and transfer to the constant humidity desiccators. The samples were then weighed at twenty-four hour intervals until constant weight was attained; at a relative humidity of 40% this required several weeks. When constant weight was attained, moisture was determined by drying at 80°. Samples which showed a loss in weight were replaced in the same desiccators and weighed at intervals to observe if the water lost during the heating would be regained. The losses

TABLE I.—WATER CONTENT OF CAFFEINE DRIED AT VARIOUS HUMIDITIES

Approximate Relative Humidity	% Water		Moles Water Present		Moles Water Regained	
	A	B	A	B	A	B
0	0	0	0	0	0	0
20	0	0	0	0	0	0
40	0	0	0	0	0	0
60	6.95	6.87	0.819	0.809	0	0
80	6.95	6.89	0.819	0.812	0	0
97	6.93	6.95	0.816	0.819	0.816	0.819
100	7.08	6.97	0.834	0.821	0.834	0.821

in weight, calculated as per cent water and as moles of water per mole of caffeine, and the moles of water regained when replaced in the constant humidity desiccators, are presented in Table I.

To study the loss of water of hydration under more practical conditions duplicate portions of about 2 Gm. each of samples "A" and "B" were weighed into open dishes and placed in a room having a constant temperature of 21° and a constant relative humidity of 64%. These samples were weighed at intervals over a twelve-week period. The loss in weight calculated as per cent water is tabulated in Table II. When constant weight was attained the samples were dried at 80°; they showed no further loss in weight. These observations indicate that under ideal storage conditions, e.g., 21°, and 64% relative humidity, Caffeine will lose its water of hydration.

TABLE II.—WATER CONTENT OF CAFFEINE DRIED AT 64% RELATIVE HUMIDITY AND 21°

	Sample "A" (1)	Sample "A" (2)	Sample "B" (1)	Sample "B" (2)
Original % of water	7.05	7.10	6.96	6.99
% water after 3 days	7.03	7.09	6.96	6.99
% Water after 10 days	6.46	6.94	6.96	6.89
% Water after 2 wk.	4.17	4.88	6.96	6.97
% Water after 3 wk.	1.46	1.91	6.43	6.47
% Water after 4 wk.	0.72	0.94	5.22	5.95
% Water after 5 wk.	0.54	0.62	4.26	4.44
% Water after 6 wk.	0.23	0.21	3.50	3.62
% Water after 7 wk.	0	0.07	3.06	3.02
% Water after 8 wk.	0	0	2.52	2.56
% Water after 9 wk.	0	0	1.06	1.16
% Water after 10 wk.	0	0	0.35	0.63
% Water after 11 wk.	0	0	0.10	0.18
% Water after 12 wk.	0	0	0	0
Loss when dried at 80°	0	0	0	0

Since one could attribute the fact that neither sample "A" nor sample "B" regained its water content, at relative humidities of 60% and 80%, to possible crystal modification during heating, the samples were rendered anhydrous by drying over P_2O_5 at room temperature, then placed in the 60% and 80% constant humidity desiccators; neither sample regained its water when allowed to remain in the desiccators for one month.

To refute a statement of Tassily (3) that when dried at temperatures as high as 150° caffeine still retains some of its water of hydration, samples "A" and "B" were analyzed for nitrogen (Kjeldahl) after being dried to constant weight at 80°.

Anal.—Calcd. for nitrogen (Kjeldahl): 28.85%; Found: For sample A, 28.70%; for sample B, 28.72%.

It must be concluded that, when dried at 80°, crystalline caffeine becomes anhydrous.

To determine the effect of drying caffeine at various temperatures, four samples from different sources have been dried at 80°, 100° and 105°. The procedure was to weigh approximately 1 Gm. of caffeine into a tared dish, then dry for two hours at 80°. To be certain that samples had reached constant weight at this temperature they were dried for an additional hour. Samples were then dried for two one-hour periods at 100° followed by two one-hour periods at 105° and finally for eighteen hours at the higher temperature. The results are tabulated in Table III in terms of milligrams lost per gram of caffeine. After prolonged heating at 105°, sublimed caffeine was observed around the air vents of the oven.

A check has also been made on the loss of caffeine when heated at 80° for a prolonged period. A sample was heated for four days at 80° and was observed to have lost a maximum of 3.3 mg./Gm.

TABLE III.—LOSS IN WEIGHT OF CAFFEINE ON DRYING AT 80°, 100° AND 105°

	Loss			
Conditions	Sample A, Mg./Gm.	Sample B, Mg./Gm.	Sample C, Mg./Gm.	Sample D, Mg./Gm.
During an additional hour at 80°	0	0	0	0
1 hr. at 100°	0.2	0.4	0.2	0.2
2nd hr. at 100°	0	0	0	0
1st hr. at 105°	1.0	0.5	0.3	0.5
2nd hr. at 105°	1.0	0.4	0.3	0.7
18 hr. at 105°	4.7	7.2	4.2	4.8

CONCLUSION

1. The monohydrate of caffeine as described by U. S. P. XII does not exist as a stable hydrate under normal conditions.

2. The hydrate of caffeine which is stable at high relative humidities may be represented as containing $^5/_6$ moles of water per mole of caffeine.

3. Approximately the same percentage moisture is obtained when crystalline caffeine is dried at 100° as when dried at 80°.

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Aminoalcohols. XV. *p*-Aminopropadrine*

By WALTER H. HARTUNG and CARROLL P. FOSTER

INTRODUCED into clinical practice as "Ephetonal" without adequate pharmacological background (1) *p*-aminoephedrine (2) is reported to possess activity similar, in many respects, to its *p*-hydroxy isoster. It is well tolerated and differs from ephedrine in that it is appreciably less toxic (3), does not cause tachyphylaxis and apparently causes a rise in the blood sugar level (4). "Ephetonal" is apparently the only aromatic amino compound of the ephedrine type which has received any pharmacological attention, although other members are described. To *m*-aminoephedrine and *p*-aminonorephedrine are ascribed therapeutic properties which compare favorably with those of ephedrine (5). Other compounds are *p*-amino-N-methylephedrine and *p*-aminophenyl-1-methylamino-2-ethanol-1 (6).

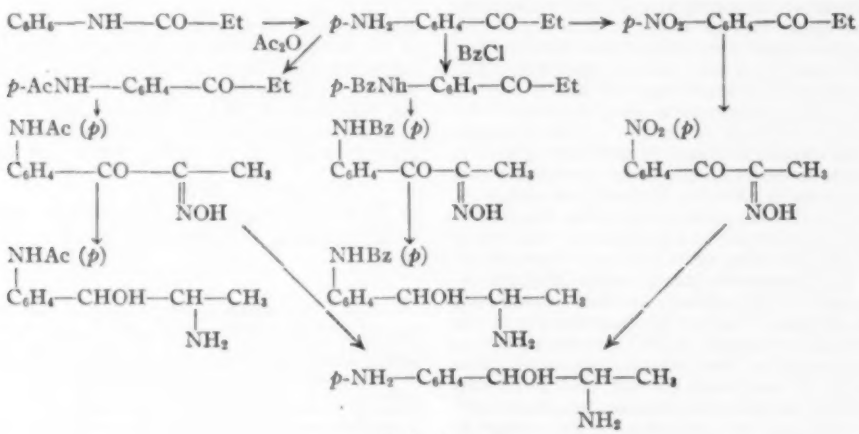
According to the disclosures, the *p*-amino compounds were obtained by nitrating the aminoalcohol, $C_6H_5—CHOH—CH(N(R')R'')—R$, and reducing. For example, from 20 Gm. norephedrine (propadrine) 6 to 7 Gm. *p*-nitronorephedrine may be expected; on reduction in the presence of palladium oxide, 1.5 Gm. of the dihydrochloride of *p*-aminonorephedrine is obtained; thus from 0.13 mole starting material is obtained a yield of 0.006 mole of final product (5a).

In view of the favorable physiological activity of *p*-aminoephedrine, as well as the high activity of amino compounds in general, a study of the change produced in the biological properties of propadrine by the introduction of an amino group into the *p*-position of the molecule was undertaken. For this purpose, a new synthesis, starting with *p*-nitropropiophenone, was investigated. Unfortunately, this desirable ketone itself is difficult to obtain and, accordingly, a second method, starting with *p*-acylaminopropiophenone, was explored. Both ketones may be readily converted into their corresponding oximino intermediates, and these then may be reduced to the corresponding amino alcohols.

The reactions employed are indicated in Equation I.

Commanducci and Pescitelli (7) describe the nitration of propiophenone with "104 per cent" nitric acid at temperatures above 40° C., and they report the isolation of a product melting at 114°, which they called *p*-nitropropiophenone, but they give no further characterization of their product. Numerous experimental attempts in these Laboratories indicate that the conditions stated by the Italian workers are impossible, and it is now established that *p*-nitropropiophenone melts at 90–91° C. and not at 114°.

In red fuming nitric acid phenyl ketones do not nitrate (8), and during the course of nitration with colorless fuming nitric acid, sp. gr. 1.48, a mixture of *o*- and *m*-nitropropiophenones may be obtained if the temperature of the reaction is kept low; but never has a trace of the *p*-isomer been



Equation I

* Received April 9, 1945, from the Research Laboratories, School of Pharmacy, University of Maryland, Baltimore.

rise above 25° C., for then the reaction may easily get out of control, red fumes belch forth and the final product, in fair yields, is usually *m*-nitrobenzoic acid. In the light of these results, consistently duplicated, the use of a temperature above 40° C., as directed by Commanducci and Pescitelli, is incomprehensible.

p-Nitropropiophenone has now been synthesized, from *p*-aminopropiophenone, and characterized. Unfortunately the yields are low.

p-Aminopropiophenone is described by Kunckell (9) and by Derrick and Bornemann (10). These authors employed an adaptation of the Fries reaction, allowing propionyl chloride to react with acetanilide. Their conditions do not preclude contamination from *p*-aminoacetophenone derivatives, a fact which has been confirmed. A purer product is obtained if propionanilide is used. The yields of *p*-aminopropiophenone averaged somewhat less than one-third of theoretical.

The pharmacological properties of *p*-aminopropadrine are being studied by Dr. Karl Beyer of Sharp and Dohme, who will report in greater detail at another time. It may be mentioned at this time, however, that the compound does exhibit considerable parallelism with its isosteric *p*-hydroxypropadrine. It exhibits a high degree of circulatory activity and does not give rise to tachyphylaxis.

EXPERIMENTAL

***p*-Aminopropiophenone.**—The procedure, based on that of Kunckell (9) and of Derrick and Bornemann (10), but differing in details, found to give best consistent yields is as follows: In a 5-L., round-bottom, three-neck flask, equipped with sealed mechanical stirrer, dropping funnel, and reflux condenser (the upper end of which is attached to an HCl-absorption train), are placed 140 Gm. aniline (1.5 moles) and 1600 ml. carbon bisulfide. The flask is immersed in a water bath at 20–25° C.; to the rapidly stirred contents is slowly added 277.5 Gm. propionyl chloride (3 moles). The dropping funnel is now replaced with a funnel having a wide stem (suitable for addition of powder) through which 400 Gm. aluminum chloride (3 moles) is added, in approximately 15-Gm. portions and over the period of about an hour, to the rapidly agitated contents of the flask. During this reaction it is desirable to have the HCl-absorption train under slightly reduced pressure. The mixture is then stirred and heated on the water bath to gentle refluxing of the solvent until the evolution of HCl practically ceases (about five hours). The stirred mixture is then cooled on an ice bath and 100 ml. cold 10% HCl added dropwise, keeping the temperature below 30° C.; this is followed by sufficient ice and water to decompose the complex completely. The heterogeneous mixture is now filtered through a Büchner funnel; the small amount of solid material is *p*-propionylaminopropiophenone, which after recrystallization from hot water melts at 149–150° C.; the filtrate, placed in a separatory funnel, separates into three layers.

The lowest layer is carbon bisulfide which, after volatilization, leaves an additional crop of 6 to 9 Gm. *p*-propionylaminopropiophenone. The middle or aqueous layer, when basified, liberates a solid weighing 1.5 to 3 Gm.; this is *p*-aminopropiophenone. The upper layer, primarily organic, is thick, orange-black in color, contains most of the product as free amine or its N-propionyl derivative; it is refluxed directly for several hours in 500 ml. 20% HCl. After hydrolysis is complete, decolorizing carbon is added to the hot acid solution followed by an equal volume of water; the solution is again brought to boiling and allowed to cool before filtering. The cold filtrate is basified and the liberated *p*-aminopropiophenone, weighing 30 to 37 Gm., is recrystallized from 40% ethanol. The combined yield is about 30%.

The purified *p*-aminopropiophenone appears as yellow crystals melting at 140° C. The hydrochloride melts at 198–199° C. The oxime melts at 153–154° C. Acetylization forms *p*-acetylaminopropiophenone melting at 172–173° C.; found N (Kjeldahl), 7.32% and 7.16%;[1] calculated N for $C_{11}H_{13}O_2N$, 7.29%; oxime of acetyl derivative melts at 156–157° C. (dec.).

p-Benzoylaminopropiophenone, formed in yields of 95%, melts at 187–188° C.; found N (Kjeldahl), 5.59% and 5.57%; calculated N for $C_{16}H_{15}O_2N$, 5.53%; the oxime melts at 164–165° C. (dec.).

***p*-Propionylphenyldiazonium** borofluoride is obtained in yields of 85% by treating *p*-aminopropiophenone with fluoboric acid according to the method of Starkey (11). The compound decomposes at 85° C., but like other diazonium borofluorides is quite stable at room temperature. Hydrolysis in boiling water yields *p*-hydroxypropiophenone, melting at 147–148° C.

***p*-Nitropropiophenone** is obtained by replacing the diazonium group with the nitro group (11, 12). The yield, unfortunately, is rarely above 25%. The product, recrystallized from hot water and decolorized with charcoal, appears as pale yellow crystals, melting at 90–91° C. Found N (Kjeldahl), 7.72% and 7.75%; calculated N for $C_9H_9O_3N$, 7.82%. Oxidation with permanganate forms *p*-nitrobenzoic acid, melting at 240° C. The oxime, recrystallized from dilute ethanol, melts at 124° C. (dec.).[2]

Oximino ketones.—The synthesis of compounds of general formula Ar—CO—C(=NOH)—R and their use as intermediates in preparing primary amino analogs of the ephedrine bases has been described (13). By following established procedures the oximinoketones listed in Table I were obtained. The slightly alkaline solutions of the oximino ketones, treated with hydroxylamine, formed excellent yields of the glyoximes, Ar—C(=NOH)—C(=NOH)—CH_3, which are also listed in Table I. These glyoximes formed colored, insoluble chelates with ammoniacal solutions of nickel salts.

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[1] Derrick and Bornemann (10) allowed propionyl chloride to react with acetanilide. When their directions are followed, a product melting at 165–168° C. is obtained, as they describe, which they call *p*-acetaminopropiophenone. Analysis of this product shows N (Kjeldahl) 6.87%; calculated N for $CH_3CO—NH—C_6H_4—CO—C_2H_5$, 7.33%; calculated N for $C_2H_5CO—NH—C_6H_4—CO—CH_3$, 6.82%.

Another possible product with Derrick and Bornemann's conditions is *p*-propionylaminoacetophenone. This compound, prepared in these Laboratories by E. C. Weinbach, melts at 131° C.

[2] The method of synthesis, combined with the analysis and oxidation, leaves no doubt about the identity of *p*-nitropropiophenone. As already indicated, the characterization by Commanducci and Pescitelli (7) is erroneous.

Reduction.—The catalytic hydrogenation of the α-oximinoethyl ketones was carried out in absolute ethanolic 2*N* HCl by means of palladium on charcoal (13, 14). Although reduction proceeded at ordinary pressures, the use of hydrogen at 10 to 20 atmospheres hastened the process and, as a rule, led to purer product.

***p*-Aminopropadrine.**—Three grams (0.015 mole) *p*-nitrophenyl α-oximinoethyl ketone and the catalyst (prepared from 3 gr. charcoal and 0.3 gr. $PdCl_2$) were suspended in 90 ml. absolute ethanolic 2*N* HCl, and the mixture shaken in an atmosphere of hydrogen at ordinary pressure; only five-sixths of the calculated hydrogen was taken up when reduction stopped completely. The catalyst was filtered out and the filtrate was evaporated to dryness in an evacuated desiccator over sulfuric acid, calcium chloride, and soda lime; the residue was gummy and hard to purify. This substance darkened on heating but did not melt below 300° C.; dissolved in water, it reduced Fehling's solution and on treatment with ammonia water color characteristic of pyrazine formation developed. Since this behavior is quite characteristic for α-amino ketones, it is believed that the product was the dihydrochloride of *p*-aminophenyl α-aminoethyl ketone. Found Cl (Volhard), 29.6% and 29.7%; calculated Cl for $C_9H_{12}ON_2 \cdot 2HCl$, 29.9%.

When the reduction was repeated at 15 atmospheres pressure, the reaction was complete in little more than an hour. The product, isolated as described above for the aminoketone above, did not reduce Fehling's solution, gave no evidence of dihydropyrazine formation, and melted at 192–193° C. (dec.). Oberlin (5) describes the dihydrochloride of *p*-aminonorephedrine as melting at 192–193° C. (dec.). Found Cl (Volhard), 29.62% and 29.60%; calculated Cl for $C_9H_{14}ON_2 \cdot 2HCl$, 29.71%.

***p*-Acetaminopropadrine.**—Seven grams (0.033 mole) *p*-acetaminophenyl α-oximinoethyl ketone and catalyst suspended in 100 ml. absolute 2*N* ethanolic HCl was reduced at an initial pressure of 15 atmospheres hydrogen; reduction proceeded smoothly until two-thirds the calculated H_2 was taken up (forty-eight minutes) and then stopped. The product, isolated as described above, represented a yield of 97% of the aminoketone hydrochloride, *p*-$CH_3CONH—C_6H_4—CO—CH(NH_2 \cdot HCl)—CH_3$, melting at 257–258° C. (dec.). On treatment with ammonium hydroxide, the product gave color reactions characteristic for pyrazine formation.

The aminoketone hydrochloride was dissolved in 100 ml. 3.5% HCl and reduced further with fresh catalyst, initial hydrogen pressure $6\frac{1}{2}$ atmospheres. In half an hour the calculated H_2 was taken up and further reduction ceased. The product, after removal of the solvent as described above, consisted of two components, the dihydrochloride of *p*-aminopropadrine and the monohydrochloride of *p*-acetaminopropadrine; the latter being insoluble in cold absolute ethanol could be isolated by fractional crystallization. The hydrochloride of *p*-acetaminopropadrine melts at 236–237° C. (dec.). Found Cl, 14.77% and 14.92%; calculated Cl for $C_{11}H_{16}O_2N_2 \cdot HCl$, 14.50%. In some reduction experiments complete removal of the acetyl group was observed.

***p*-Benzoylaminopropadrine.**—*p*-Benzoylaminophenyl α-oximinoethyl ketone, reduced under conditions similar to those employed for the analogous *p*-acetamino compound, also stopped at the intermediate aminoketone stage; this did not melt below 340° C., reduced Fehling's solution, and with ammonium hydroxide gave the dihydropyrazine color reaction. Further reduction in dilute HCl formed pure *p*-benzoylaminopropadrine, which was isolated as the hydrochloride, nonfusible below 340° C. and stable toward Fehling's solution. Found Cl, 11.49% and 11.48%; calculated Cl for $C_{16}H_{18}O_2N_2 \cdot HCl$, 11.58%.

TABLE I.—ARYL α-OXIMINOETHYL KETONES

p-R—C_6H_4—CO—C(=NOH)—CH_3

R =	Yield, %	M. P., ° C.	Molecular Formula	Analyses for N Glyoxime: % Found	% Calcd.	M. P. (dec.)
NO_2	70	132–133	$C_8H_8O_4N_2$	13.35 13.32	13.46	197
—$NHCOCH_3$	75	219	$C_{11}H_{12}O_3N_2$	12.69 12.75	12.72	225
—$NHCOC_6H_5$	75	209 (dec.)	$C_{18}H_{14}O_3N_2$	9.84 9.81	9.93	245

Hydrolysis of *p*-acylaminopropadrines.—In view of the fact that appreciable amounts of *p*-aminopropadrine formed during the reduction of *p*-acetaminophenyl α-aminoethyl ketone, involving removal of the acyl group, no difficulty was anticipated in hydrolyzing the *p*-acylaminopropadrine derivatives. However, side reactions, the nature of which has not been established, interfere and none of the desired product has thus far been obtained or isolated in this manner.

Half-gram *p*-acetaminopropadrine hydrochloride refluxed gently in 30 ml. 20% HCl for ninety minutes yielded yellow crystals melting 160° C. (dec.), which by the Volhard method showed the presence of 26.1% Cl; it is not unlikely that the product is impure dihydrochloride of *p*-aminophenyl-1-amino-2-chloro-1-propane, *p*-$NH_2—C_6H_4—CHCl—CH(NH_2)—CH_3 \cdot 2HCl$, calculated available Cl, 27.5%; that is, the alcoholic hydroxyl group may have been replaced by a Cl atom.

Gentle refluxing in dilute HCl had little effect on *p*-benzoylaminopropadrine; usually the starting material was largely recovered unchanged, contaminated with only small amounts of yellow crystals which could not be identified as the desired hydrolytic product. More drastic conditions gave unchanged material, some benzoic acid but nothing that could be identified as *p*-aminopropadrine. The use of concentrated HCl gave unchanged *p*-benzoylaminopropadrine, larger amounts of benzoic acid, and a resinous fraction from which no *p*-aminopropadrine could be isolated. *p*-Benzoylaminopropadrine was not affected by refluxing with a solution of sodium bicarbonate.[3]

[3] It is not presumed that there is any similarity, but this resistance of the benzoyl group to hydrolysis calls to mind Abel's difficulty [Abel, *Bull. Johns Hopkins Hosp.*, **12**, 337 (1901)] in hydrolyzing completely his polybenzoyl derivative of epinephrine.

SUMMARY

1. The synthesis of *p*-aminopropiophenone and its acylated derivatives is described.

2. *p*-Nitropropiophenone has been synthesized and characterized.

3. *p*-Nitro-, *p*-acetamino- and *p*-benzoylaminopropiophenone have been converted into their corresponding α-oximinoethyl ketones, which have been catalytically reduced to *p*-amino-, *p*-acetamino- and *p*-benzoylaminopropadrine.

4. The attempts to hydrolyze *p*-acetaminopropadrine and *p*-benzoylaminopropadrine to *p*-aminopropadrine proved unsuccessful.

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A Synthesis of *p*-Aminophenylalanine*

By ALBERT M. MATTOCKS and WALTER H. HARTUNG

A new synthesis of *p*-aminophenylalanine from *p*-nitrophenylpyruvic acid is described. Unsuccessful attempts at nitrosation of *p*-nitrobenzylmalonic ester and *p*-nitrobenzylacetoacetic ester are recorded.

p-Aminophenylalanine has not been found in nature, nor is it an amino acid that might be suspected to occur in the animal body; however, being a nitrogen analog of tyrosine, it might be metabolized in a manner similar to that of tyrosine and yet produce a different physiological action. Such action seems probable, since Selinger (1) found that *p*-aminoephedrine is chemically and physiologically isosteric with *p*-hydroxyephedrine. Hence, in order that *p*-aminophenylalanine might be available for physiological studies, its synthesis was undertaken.

Erlenmeyer and Lipp (2) nitrated phenylalanine, and isolated the *p*-nitro compound which they reduced to obtain *p*-aminophenylalanine. Friedländer (3) prepared *p*-aminophenylalanine by reduction of *p*-nitrophenyl-α-nitroacrylic acid. Takayama and Tubuku (4), in a study of the nitration of phenylalanine, obtained *p*-nitrophenylalanine in 53 per cent yield, but they did not reduce the compound.

In the present investigation *p*-nitrotoluene was condensed with ethyl oxalate to yield ethyl *p*-nitrophenylpyruvic acid, and the keto ester was converted into a corresponding α-oximino ester by condensation with hydroxylamine. Simultaneous catalytic reduction of the oximino and nitro groups produced the ethyl ester of *p*-aminophenylalanine. Attempts to prepare α-oximino-β-keto-β-(*p*-nitrophenyl)-propionic acid from appropriately substituted malonic and acetoacetic esters, according to the procedures developed by Barry (5) were unsuccessful.

EXPERIMENTAL

Nitrosation Experiments.—*p*-Nitrobenzylmalonic ester, prepared according to the procedure of Reissert (6) and by direct nitration of benzylmalonic ester, was treated with isopropylnitrite in the presence of sodium ethoxide and also with isopropylnitrite in the presence of hydrochloric acid, but nitrosation did not proceed in either case. Mixtures of products giving no consistent results on Kjeldahl analysis were obtained by these methods. Similar results were obtained by nitrosation experiments on *p*-nitrobenzylacetoacetic ester.

***p*-Nitrophenylpyruvic Acid Ethyl Ester.**—*p*-Nitrotoluene was condensed with ethyl oxalate in the presence of sodium ethoxide according to the procedure of Wislicenus and Schulz (7), except that the red sodium salt of the quinoid form was removed from the solution as such and used in this form.

* Received Aug. 23, 1945, from the Research Laboratories, School of Pharmacy, University of Maryland, Baltimore, Md.

α-Oximino-β-(*p*-nitrophenyl)-propionic Acid.—In a one-litre Erlenmeyer flask was placed a solution of 23.1 Gm. (0.1 mole) of the sodium salt of the quinoid form of *p*-nitrophenylpyruvic acid ethyl ester in 100 cc. of methyl alcohol. To this was added a solution of 13.8 Gm. (0.2 mole) of hydroxylamine hydrochloride and 27.2 Gm. (0.2 mole) of sodium acetate in 300 cc. water. After mixing the two solutions, 10 cc. glacial acetic acid was added with stirring. The reaction mixture became light yellow in color, and the flask was stoppered and allowed to stand for one hour with occasional shaking. The mixture was transferred to a distilling flask, and most of the methyl alcohol was removed at room temperature under reduced pressure. The residual aqueous mixture was cooled overnight, and a flocculent yellow precipitate was formed. The crystals were filtered off and washed with cold water, then dried over sulfuric acid. Pale yellow crystals melting at 161–162° and weighing 22.6 Gm. (90%) were obtained.

Anal.—Calcd. nitrogen for $C_{11}H_{12}O_5N_2$. 11.1%. Found: (Kjeldahl) 11.3%, 11.4%.

***p*-Aminophenylalanine Ethyl Ester Dihydrochloride.**—To 5.2 Gm. (0.021 mole) of α-oximino-β-(*p*-nitrophenyl)-propionic acid ethyl ester dissolved in 100 cc. of 50% alcohol were added 10 cc. 36% hydrochloric acid and 3 Gm. 10% palladium-5% platinum catalyst. The mixture was shaken in hydrogen at a pressure of one atmosphere, and the theoretical quantity of hydrogen, 2300 cc., was absorbed in six hours. The absorption of hydrogen was rapid at first, 1100 cc. being taken up in one hour, but the reduction became much slower as it neared completion. The mixture was filtered to remove the catalyst, and the clear filtrate was transferred to a Claisen flask. The flask and contents were warmed to 60° and the solvents removed at reduced pressure. After evaporation of solvents there remained a residue of faintly yellow crystals. These were redissolved in absolute alcohol and precipitated by the addition of isopropyl ether, then filtered out and dried. Five grams (87%) of the ethyl ester of *p*-aminophenylalanine dihydrochloride was obtained. The crystals were pale yellow and very hygroscopic.

Anal.—Calcd. nitrogen for $C_{11}H_{16}O_2N_2 \cdot 2HCl$: 9.99%. Found: (Kjeldahl) 10.1%, 10.5%.

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Determination of Magnesium in Magnesium Sulfate and Solution of Magnesium Citrate*

By SAMUEL W. GOLDSTEIN†,‡

Magnesium Citrate Solution and Magnesium Sulfate were assayed by oxine and by pyrophosphate procedures and the results compared. Precipitation of the magnesium as the oxyquinolate is recommended as the preferred method.

THE proposed new United States Pharmacopœia XIII assay for magnesium oxide in Magnesium Citrate Solution (1) is based on Berg's (2) procedure, using 8-hydroxyquinoline as modified by Bell (3). Fifty cubic centimeters of the Solution are diluted to exactly 100 cc. with water. Five cubic centimeters of this dilution are mixed with 150 cc. of distilled water at 70° to 80°. Then 1 cc. of 2 *N* ammonium chloride and 3 cc. of stronger ammonia are added and mixed, followed by 8 cc. of a 5 per cent solution of oxine in alcohol which is added slowly with constant stirring. After thirty minutes, the precipitate is washed three times by decantation and is collected on a sintered-glass filter crucible, previously dried at 100° to 105° and weighed, washed well with distilled water, and the crucible and contents are dried for two hours at 100° to 105°. The weight of magnesium oxyquinolate multiplied by 4.628 gives the equivalent of magnesium oxide in 100 cc. of the Solution.

In order to check the new method, 21

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‡ The author acknowledges the interest and advice of Dr. William F. Reindollar, Chief of the Bureau of Chemistry, Maryland State Department of Health.

commercial samples of Solution of Magnesium Citrate were assayed by the pyrophosphate procedure of the U. S. P. XII (I) and the proposed oxine method (II). The results of the analyses are given in Table I.

These results indicate that the oxine procedure yields results nearer the true values, the average being 99.92%; while the average for the results by the pyrophosphate method is 101.04%.

As a further check on the magnesium sulfate heptahydrate, 9.7941 Gm. of the salt was heated in a muffle at about 420° until no further loss in weight was noted (forty-four hours). The weight of the residual anhydrous magnesium sulfate, 4.7875 Gm., represented 48.88% of the hydrated salt. The theoretical value is 48.84%, indicating the loss of a negligible amount of water of hydration. Anhydrous magnesium sulfate, 8.608 Gm., (prepared as above) was placed in a 250-cc. volumetric flask and distilled water was added to make 250 cc. of solution at 20°. This solution was assayed by the U. S. P. XII procedure for Magnesium Sulfate using 15-cc. ali-

TABLE I

Sample	U. S. P. Method: Gm. of Pyrophosphate	U. S. P. Method: Equiv. MgO in Gm.	U. S. P. Method: % of MgO	Oxine Method: Gm. of Oxyquinolate	Oxine Method: Equiv. MgO in Gm.	Oxine Method: % of MgO
1	0.4307	0.1559	1.56	0.3334	0.03858	1.54
2	0.4383	0.1587	1.59	0.3309	0.03830	1.53
3	0.4653	0.1685	1.69	0.3530	0.04085	1.63
4	0.4447	0.1611	1.61	0.3338	0.03865	1.55
5	0.4499	0.1629	1.63	0.3364	0.03893	1.56
6	0.4496	0.1628	1.63	0.3359	0.03888	1.56
7	0.4477	0.1621	1.62	0.3389	0.03920	1.57
8	0.4543	0.1645	1.65	0.3437	0.03978	1.59
9	0.4210	0.1525	1.53	0.3202	0.03705	1.48
10	0.4431	0.1604	1.60	0.3398	0.03933	1.57
11	0.4558	0.1651	1.65	0.3466	0.04010	1.60
12	0.4489	0.1626	1.63	0.3431	0.03970	1.59
13	0.4431	0.1604	1.60	0.3416	0.03953	1.58
14	0.4417	0.1600	1.60	0.3396	0.03930	1.57
15	0.4426	0.1603	1.60	0.3403	0.03938	1.58
16	0.4590	0.1662	1.66	0.3491	0.04040	1.62
17	0.4249	0.1538	1.54	0.3259	0.03440	1.51
18	0.4921	0.1782	1.78	0.3650	0.04223	1.69
19	0.4294	0.1555	1.56	0.3281	0.03798	1.52
20	0.4372	0.1583	1.58	0.3328	0.03850	1.54
21	0.4386	0.1587	1.59	0.3354	0.03881	1.55

With method I 14 samples met the minimum requirement of 1.60 Gm. of magnesium oxide in 100 cc. of Solution of Magnesium Citrate, while 7 samples fell below the lower limit, only 2 samples dropping below 1.55 Gm. Method II showed only 4 samples meeting the minimum requirement with 6 samples dropping below 1.55 Gm.

According to the above results, method II should not replace method I unless it is shown that the results obtained by method II are in closer agreement with the true values than are those yielded by method I. To investigate this problem, the following experiments were carried out.

TABLE II.—ANALYSES OF MAGNESIUM SULFATE BY U. S. P. AND OXINE METHODS

$MgSO_4 \cdot 7H_2O$ Weighed	Gm. of Pyrophosphate	Equivalent $MgSO_4 \cdot 7H_2O$ in Gm.	% Recovery
U. S. P. Method			
1.0873	0.4965	1.0995	101.12
0.9967	0.4547	1.0070	101.03
0.9424	0.4309	0.9543	101.26
1.0658	0.4846	1.0732	100.79
1.0833	0.4943	1.0947	101.05
0.9987	0.4554	1.0085	100.98
Oxine Method			
$MgSO_4 \cdot 7H_2O$ Weighed	Gm. of Oxyquinolate	Equivalent $MgSO_4 \cdot 7H_2O$ in Gm.	% Recovery
0.2759	0.3890	0.2750	99.67
0.2597	0.3654	0.2583	99.46
0.3032	0.4274	0.3022	99.67
0.2582	0.3652	0.2582	100.00
0.2710	0.3845	0.2718	100.29
0.2653	0.3769	0.2665	100.45

EXPERIMENTAL

Magnesium sulfate heptahydrate, reagent grade, was analyzed by the U. S. P. XII pyrophosphate procedure and by the above given oxine procedure, with the use of 3 cc. of 2 *N* ammonium chloride instead of 1 cc. The first three oxyquinolate precipitates were heated at 100–105° for one and a half hours and then for three half-hour periods. The next three were heated at 100–105° for one two-hour and two half-hour periods. In both cases constant weight was attained in two hours. The results are given in Table II.

quots, containing 0.5165 Gm. of the anhydrous salt. The oxine procedure was carried out using 5-cc. aliquots, containing 0.1722 Gm. of the anhydrous salt, and using 4 cc. of 2 *N* ammonium chloride and 10 cc. of the 5% oxine reagent. The results of analyses in triplicate were 101.24% recovery by the pyrophosphate method, and 99.55% recovery by the oxine method.

The above results obtained in the analyses of hydrated and anhydrous magnesium sulfate indicate that the pyrophosphate assay for Magnesium Sulfate in the U. S. P. XII should be replaced by the oxine assay. The following procedure is recommended:

Transfer the accurately weighed Magnesium Sulfate obtained in the test for *loss on drying* to a 100-cc. volumetric flask and add water to make 100 cc. of solution. Transfer exactly 25 cc. of this solution to a beaker containing 130 cc. of water heated to about 80°, add 4 cc. of ammonium chloride T. S. and then 3 cc. of stronger ammonia T. S. Mix thoroughly and add dropwise, with stirring, 8 cc. of 8-hydroxyquinoline T. S. (5% in alcohol). After standing for thirty minutes, pour off the supernatant liquid through a sintered-glass filter crucible, previously dried at 100° to 105° and weighed. Wash the precipitate three times by decantation, and then transfer it completely to the filter and wash thoroughly with water. Dry the crucible and contents for two hours at 100° to 105°, cool, and weigh. The weight of magnesium oxyquinolate, multiplied by 1.381, indicates the equivalent weight of $MgSO_4$ in the weighed sample.

A solution of magnesium sulfate was prepared by transferring 12.154 Gm. of anhydrous salt to a 100-cc. volumetric flask and adding water to make 100 cc. From this solution two simulated solutions of magnesium citrate were prepared by measuring from a burette into 100-cc. volumetric flasks 46.67 cc. and 39.30 cc., containing 5.6723 Gm. of $MgSO_4$ and 4.7765 Gm. of $MgSO_4$ equivalent to 1.90 Gm. of MgO and 1.60 Gm. of MgO, respectively. To each flask were added 9.4 Gm. of citric acid and 14.6 Gm. of sucrose and enough water to make 100 cc. of solution. These two solutions were assayed by the U. S. P. XII method (I) and by the oxine method (II). The oxyquinolate precipitate did not seem to reach a reasonably constant weight in two hours at 100–105° and the heating was continued as indicated in Table III.

The results indicate that the oxyquinolate precipitate and crucible should be heated for at least three hours instead of two hours as stated in the proposed method, and it is recommended that this change be made. The values recorded after heating for three hours are used in Table IV showing comparative results of analyses of the simplified magnesium citrate solutions by methods I and II.

TABLE III.—RELATIONSHIP OF RESULTS BY OXINE METHOD TO LENGTH OF HEATING PERIOD

Sample No.	Calculated Gm. of MgO in 100 Cc. of Solution	Results Expressed as Gm. of MgO/100 Cc. and % Recovery — 2 Hr. at 100–105°		2½ Hr. at 100–105°		3 Hr. at 100–105°		3½ Hr. at 100–105°	
1	1.90	1.9077	100.40	1.9067	100.35	1.9035	100.18	1.8998	99.99
2		1.9067	100.35	1.9044	100.23	1.9012	100.06	1.8970	99.84
3		1.8998	99.99	....	....	1.8956	99.77	1.8952	99.75
4		1.8998	99.99	....	....	1.8933	99.65	1.8933	99.65
5		1.9049	100.26	....	....	1.8961	99.79	1.8952	99.75
6		....	....	....	....	1.9095	100.50	1.9086	100.45
7		....	....	....	....	1.9058	100.31	1.9053	100.28
8		....	....	....	....	1.9077	100.40	1.9072	100.38
9	1.60	1.6105	100.66	....	....	1.6078	100.48	1.6064	100.40
10		1.6129	100.80	....	....	1.6096	100.60	1.6082	100.51
11		1.6166	101.04	....	....	1.6092	100.57	1.6073	100.46
12		1.6092	100.57	....	....	1.5999	99.99	1.5967	99.79
13		1.6133	100.83	1.6119	100.75	....	....	1.6018	100.11
14		1.6096	100.60	1.6073	100.46	....	....	1.5957	99.73
15		1.6175	101.09	1.6142	100.89	....	....	1.6013	100.08
16		1.6119	100.75	1.6082	100.51	....	....	1.5925	99.53
17		....	....	....	....	1.5934	99.59	1.5920	99.50
18		....	....	....	....	1.5930	99.56	1.5916	99.47
19		....	....	....	....	1.6004	100.02	1.6004	100.02
Av.			100.56		100.53		100.10		99.98

The average values of 1.946 Gm. and 1.638 Gm. per 100 cc. obtained by method I are 2.42% and 2.37% above the respective calculated values. The average values of 1.9025 Gm. and 1.6014 Gm. per 100 cc. obtained by method II are 0.13% and 0.09% above the respective calculated values. Bell (3) reported analyses by methods I and II of two standard simplified magnesium citrate solutions, prepared essentially as indicated above. His results by method I were 3.53% and 2.98% above the calculated values; while his results by method II were 0.88% and 0.83% above the calculated values. When the values obtained after heating the oxyquinolate precipitate for two hours are substituted for those in the above table, the average values in the present case by method II are 0.21% and 0.81% above the respective calculated values.

The work here reported is in substantial agreement with the findings of Bell (3) and emphasizes the superiority of the proposed oxine procedure over the pyrophosphate procedure for the determination of magnesium in Solution of Magnesium Citrate. It also indicates that those manufacturers of Solution of Magnesium Citrate who have been aiming successfully at the minimum magnesium oxide content for their preparations will find it necessary to raise their sights slightly to make a product that will meet the minimum requirement when assayed by the proposed method.

TABLE IV

Calculated Gm. of MgO in 100 Cc. of Solution	U. S. P. Method			Oxine Method		
	Gm. of Pyrophosphate	Equivalent MgO in Gm.	MgO in Gm./100 Cc.	Gm. of Oxyquinolate	Equivalent MgO in Gm.	MgO in Gm./100 Cc.
1.90	0.5366	0.1943	1.94	0.4113	0.04759	1.90
	0.5375	0.1946	1.95	0.4108	0.04753	1.90
	0.5375	0.1946	1.95	0.4096	0.04739	1.90
	0.5398	0.1954	1.95	0.4091	0.04733	1.89
	0.5360	0.1941	1.94	0.4097	0.04740	1.90
	....	....	..	0.4126	0.04774	1.91
	....	....	..	0.4118	0.04765	1.91
	....	....	..	0.4122	0.04769	1.91
1.60	0.4522	0.1637	1.64	0.3474	0.04019	1.61
	0.4528	0.1640	1.64	0.3478	0.04024	1.61
	0.4532	0.1641	1.64	0.3477	0.04023	1.61
	0.4533	0.1641	1.64	0.3457	0.04000	1.60
	0.4506	0.1632	1.63	0.3443	0.03984	1.59
	....	....	..	0.3442	0.03982	1.59
	....	....	..	0.3458	0.04001	1.60

SUMMARY

1. Results obtained in the analyses of Magnesium Sulfate and Solution of Magnesium Citrate by oxine procedures have been tabulated and compared with the corresponding results obtained by the U. S. P. methods.

2. The oxine method of assay for magnesium has been shown to be more accurate than the pyrophosphate procedures of the U. S. P. XII for Magnesium Sulfate and Solution of Magnesium Citrate.

3. A procedure for the assay of $MgSO_4$ in Magnesium Sulfate U. S. P. based on the oxine method has been described and is recommended.

4. It has been shown that the oxyquinolate precipitate obtained in the proposed new U. S. P. assay should be heated for a longer period at 100° to 105° than the directed two hours, and that heating for three hours gives more accurate results. This change in the procedure is recommended.

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Comparative Analyses of Normal and Tetraploid *Datura Stramonium* and *Datura Tatula**

By ORVILLE H. MILLER† and LOUIS FISCHER‡

Tetraploids of *Datura stramonium* and *Datura tatula* were produced by colchicine treatment and confirmed by chromosome counts. Normal and tetraploid plants did not differ appreciably in moisture, ash, and acid-insoluble ash content. Total alkaloid content, however, was generally higher in the tetraploids, particularly in the leaves, stems, and seeds. The proportions of alkaloids were not significantly altered. Both normal and tetraploid leaves contained chiefly hyoscyamine, a small amount of scopolamine, and little or no atropine. *Datura* tetraploids are self-reproducing and have a higher alkaloid content and much larger leaves than the normal plants and therefore should be of commercial value.

COLCHICINE-INDUCED tetraploids of *Datura stramonium*, with a doubled chromosome number, have been produced on several occasions by Blakeslee and associates (1–3) for the purpose of genetic studies. Shortly before this investigation was completed Rowson (4), in a brief note, reported that he had found approximately a doubling of the alkaloid content in the leaves of tetraploid *D. stramonium* and *D. tatula;* while the proportions of the individual alkaloids present remained unchanged, and that he would publish his findings in detail when finished. Since *D. stramonium* amd *D. tatula* are of considerable value in medicine and a source of the alkaloids atropine and hyoscyamine, the effect of tetraploidy on the constituents of the two plants should be of interest. *D. tatula* has formerly been classified as a distinct species, but there is now genetic evidence to indicate that it is but a variety of *D. stramonium* (5–7).

Tetraploids of *D. stramonium* and *D. tatula* were produced by soaking their seeds in colchicine solutions and the plants grown from these treated seeds were used as a source of seed for succeeding generations. Normal, diploid plants were grown at the same time to serve as controls.

The diploids and tetraploids were then compared as to the moisture content of the fresh leaves including the petioles, total ash and acid-insoluble ash content of leaf blades, petioles and stems; total alkaloids in leaf blades, petioles, stems, roots, immature capsules and ripe seeds; and the composition of the alkaloid mixture in the leaf blades.

EXPERIMENTAL[1]

The tetraploid *Datura stramonium* and *Datura tatula* used for comparison with the normal or diploid plants were produced by soaking seeds of each in colchicine solutions prior to planting, as described by Blakeslee and Avery (1). In order to eliminate possible differences occurring in strains of the same variety, seeds were first gathered from a single plant of each and then these seeds were used as a source of both normal and tetraploid material. A portion of these seeds was soaked for a five-day period in 0.5% and 1.0% solutions of colchicine, then planted in flats.

Seedlings with a stunted appearance and with stems enlarged at the base were selected for transplantation when 3 to 4 inches high. As the plants matured the tetraploids could be detected by the thicker and darker green leaves. Later, when blossoms appeared they were further identified by pollen comparisons. According to Blakeslee and Avery (1), the pollen of the tetraploids has twice the volume of normal pollen, resulting in an apparent increase in diameter of approximately one-fourth. A plant of each variety showing pollen most uniformly of twice the volume was selected as a source of seed for a second generation and these second or third generation plants were used for collection of material for analysis. Plants grown directly from colchicine-treated seeds often contain mixed diploid and tetraploid and even octaploid tissues and therefore were not suitable for comparative purposes. By using second and third generations, plants uniformly tetraploid were obtained.

In over-all size there was little difference between the diploid and tetraploid plants of the same variety. Individual plants of *D. stramonium* ranged from 2 to 4 feet in height and those of *D. tatula* from 3 to 7 feet. The stems of the tetraploids were generally somewhat thicker and more succulent. The diploid stems rapidly became woody as they matured, while a corresponding hardening in the tetraploids took place slowly over a longer period. This difference in rate of maturity was also strikingly demonstrated by the flowering period. Flowers ap-

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peared on both diploids and tetraploids when the plants were about 10 inches high and continued to appear during several months of rapid growth. Then as the plants ceased this rapid growth the diploids no longer produced flowers but rapidly developed seed capsules and within a month their leaves became yellow and most of them dropped. Meanwhile, the tetraploids continued to grow slowly, the capsules matured gradually and the plants retained their leaves and continued to flower until early winter. In several instances tetraploids were allowed to grow through the winter, and after dropping many of their leaves and occasionally some of their terminal branches, again set forth axillary shoots and grew new leaves and flowers in the manner of a biennial while diploid controls showed no sign of revival.

TABLE I.—COMPARATIVE WEIGHTS OF THE LEAVES AND PETIOLES OF DIPLOID AND TETRAPLOID *D. stramonium* AND *D. tatula*

	D. stramonium		*D. tatula*	
	Diploid	Tetraploid	Diploid	Tetraploid
Fresh				
Wt. 10 whole leaves, Gm.	118	275	126	290
Wt. 10 petioles only, Gm.	24	50	25	55
Wt. 10 leaf blades only, Gm.	94	225	101	235
Dried				
Wt. 10 whole leaves, Gm.	14.50	30.15	16.00	32.60
Wt. 10 petioles only, Gm.	1.80	4.45	1.90	4.90
Wt. 10 leaf blades only, Gm.	12.70	25.70	14.10	27.70
Amount of petiole in dried whole leaf, %	12.4	14.8	11.9	15.0
Loss on drying petiole, %	92.5	91.1	92.4	91.1
Loss on drying leaf blade, %	86.5	88.6	86.0	88.2
Loss on drying whole leaf, %	87.7	89.0	87.3	88.8

The leaves of the tetraploids of each variety differed markedly in size from those of the diploids. The tetraploid leaves were much larger, about twice the size, and were considerably thicker. Leaves with a maximum breadth of 14 to 16 inches were commonly encountered on tetraploid *D. tatula* grown in rich soil. As a means of comparison, ten representative leaves and their petioles were gathered from diploid and tetraploid plants and weighed both fresh and after drying to constant weight at 100° C. The larger, fully developed leaves were selected, but no attempt was made to pick the largest ones, but rather average full-grown leaves. The results appear in Table I. The petioles were removed from the leaf blades and weighed separately to furnish data on the relative weight of each part composing the whole leaf. The petioles of the tetraploids were particularly large, as shown by Table I, and their contribution to the alkaloid content of the whole leaf was deemed worthy of study. Subsequent analytical results showed that the petioles of both diploids and tetraploids in general contained an alkaloid content well above that of the leaf blades alone.

The increase in leaf size of the tetraploids did not result in any marked increase in yield of leaf per plant, since this factor was offset by there being only about half as many leaves on a plant of the same size. The chief advantage accruing from the increased leaf size would be the saving in time required to pick a given weight of leaf. The tetraploid leaves were also much darker green than the diploid leaves, probably because of the increased thickness and lessened transparency.

Flower size varied somewhat with individual blossoms both on the same and different plants, but in all cases the average size of tetraploid blossoms was distinctly larger and served as an additional indication of tetraploidy.

Another distinctive feature of the tetraploids was the smaller, nearly spherical seed capsules as contrasted to the elongated egg-shaped capsules of the diploids. The tetraploid seeds appeared to be of about twice the volume of the diploid seed and less than half the number per capsule. The comparative weights of the seeds are given in Table II.

As a final confirmation of tetraploidy and to eliminate unbalanced mutants with an excess or deficiency of one or more individual chromosomes, chromosome counts were made on pollen-mother-cells and root tips of both diploids and tetraploids. Blakeslee and associates (2, 3, 8) have found such

TABLE II.—WEIGHT OF 100 AIR-DRIED SEEDS

Diploid *D. stramonium*	0.955 Gm.
Tetraploid *D. stramonium*	1.545 Gm.
Diploid *D. tatula*	0.975 Gm.
Tetraploid *D. tatula*	1.635 Gm.

forms to occur commonly in colchicine-induced tetraploids and even occasionally in untreated diploids. The chromosome numbers of the plants used for comparison were 24 and 48 for diploids and tetraploids, respectively.

Culture and Collection of Material for Analysis.—Diploids and tetraploids of both *D. stramonium* and *D. tatula* were grown in adjacent rows on the same plot at Millbrae, Calif., a few miles south of San Francisco. All the seeds were planted at the same time early in May, 1943. Cultivation and watering were equalized as much as possible. In this manner, climatic and nutritional factors were kept as nearly identical as possible so that they would equally affect growth and alkaloid production and not alter the relative ratios between the four kinds. Except for occasional morning fogs the weather was clear and warm and very little rain fell throughout the growing season. The plants were watered once or twice weekly as needed. Approximately one hundred plants of each kind were grown and about one-third of each group were used for each of three collections. Flower buds and root tips were gathered from a number of the plants of each kind and used for the chromosome counts. The first collection of material for analysis was made on July 23, 1943, about three months after the first

appearance of seedlings. The weather was cool because of a heavy night and morning fog. At this time all the plants were still growing rapidly with numerous blossoms and occasional immature capsules that had developed from the earliest blossoms. Leaf blades and petioles were collected separately from about thirty plants of each lot, and from these plants five or six of each lot were pulled up and divided into roots, stems, and immature capsules; the other plants were left with their remaining leaves as a source of seed. The large stems and roots were sliced and the capsules split in half to facilitate drying. Within several hours after gathering, the material was spread out in a drier with circulating air maintained at 55° to 60° C. and dried for three days. Then the dried material was ground to a No. 20 powder and stored in airtight containers.

A second collection was made ten days later, on August 2, a hot, dry day. At this time the plants had ceased their rapid growth and the diploid plants bore fewer blossoms and a number of capsules in various stages of development. The tetraploids still bore many blossoms and their capsules were smaller, fewer in number, and less developed than those of the diploids. The material was gathered, dried, and ground in the same manner as that of the previous collection.

A third and final collection was made after another ten-day period, on August 12, a warm, partly cloudy day. By this time the diploids bore very few or no blossoms, a number of their capsules were quite mature, and a few of the lower leaves were beginning to turn brown. In contrast, the tetraploids still bore many blossoms, the capsules were but partly developed, and there was no browning of the leaves. Again, the material was gathered, dried, and ground as before.

Seeds were collected at intervals as they ripened from the remaining partly denuded plants. Practically all the diploid seed had ripened by the middle of September, but additional seed was collected from the tetraploids until the middle of November when damp weather caused the remaining seed to mold in the capsules. Even at this late date blossoms and buds were still to be found on the tetraploids. As the seeds were collected they were air dried for two weeks by spreading them out in a warm place (about 30° C.). When dry they were added to the other seed of the same kind to give a single collection of each.

As a result of this collection procedure, three individual collections of the leaf blades, petioles, immature capsules and their contents, stems, and roots from each of diploid and tetraploid plants of *D. stramonium* and *D. tatula* were obtained, representing the various plant parts at three different growth phases. In addition, composite seed collections from each of the four kinds were gathered as the seed ripened.

The coarsely ground samples, No. 20 powder, were thoroughly mixed, aliquots were taken and ground to a No. 40 powder and this material used for subsequent analyses. The ripe seeds contained a large percentage of fixed oil and could not be ground as described above. Their comminution is described later in the method of their assay.

Moisture and Ash Determinations.—The moisture content of the fresh leaves and petioles of *D. stramonium* and *D. tatula* is given in Table I. In order to correct succeeding analyses for variations in moisture content of the dried and ground samples, moisture determinations were also made on all these samples. Two-gram samples were weighed in platinum dishes, dried overnight at 100° C., and the loss in weight was calculated as per cent moisture. The results of these determinations were then used to calculate subsequent analytical data to a moisture-free basis.

Since the U. S. P. XII specifies a limit of 4% acid-insoluble ash for *Stramonium*, a comparison was made of the total ash and acid-insoluble ash content of the diploid and tetraploid *Daturas* to see what effect, if any, accompanies tetraploidy. The ash content was determined only in those portions of the plant occurring in official *Stramonium*, namely, the leaf blades, petioles, and stems. Samples from each collection were analyzed also to show any effect of the maturity of the plant on the ash content.

The residues remaining from the moisture determinations were ashed and weighed to give the total ash. This ash was then used for the acid-insoluble ash determination following the U. S. P. XII procedure (9). The results of these analyses appear in Table III.

TABLE III.—ASH CONTENT OF DIPLOID AND TETRAPLOID *D. stramonium* AND *D. Tatula*

		Leaf Blade		Petiole		Stem	
	Collection	Total Ash	Acid-Insol. Ash	Total Ash	Acid-Insol. Ash	Total Ash	Acid-Insol. Ash
		% Calculated to Dry Basis					
Diploid *D. stramonium*	1	19.30	0.89	29.08	0.15	11.90	0.05
	2	17.75	1.13	25.98	0.23	9.19	0.03
	3	19.87	1.34	23.90	0.27	8.11	0.04
Tetraploid *D. stramonium*	1	20.02	0.82	28.46	0.13	11.25	0.03
	2	15.87	0.73	24.15	0.16	9.08	0.02
	3	17.05	0.87	21.95	0.24	6.86	0.01
Diploid *D. tatula*	1	18.94	0.92	26.89	0.18	9.14	0.04
	2	17.06	0.95	28.07	0.20	11.72	0.02
	3	19.22	0.93	27.40	0.22	9.05	0.02
Tetraploid *D. tatula*	1	21.35	0.90	25.53	0.12	11.18	0.04
	2	18.67	0.78	26.19	0.13	9.62	0.02
	3	20.06	0.95	25.42	0.21	6.87	0.01

Total Alkaloid Assays.—All the collected material was assayed by the U. S. P. XII method for Stramonium (10) in order to determine the relative total alkaloid content of diploid and tetraploid *D. stramonium* and *D. tatula*.

The high fixed oil content of the ripe seeds required modifications of the foregoing procedure. The seeds were coarsely ground, just sufficient to break up the testa and contents into several fragments. This coarse powder was macerated and extracted with ether in the same manner as the other samples, then the practically oil-free residue was removed from the extraction thimbles, air dried and reground to a No. 40 powder and again macerated and extracted. The two ether extracts were combined and extracted with half-normal acid and the alkaloids determined as outlined in the of-

TABLE IV.—TOTAL ALKALOID CONTENT OF DIPLOID AND TETRAPLOID *D. stramonium* AND *D. tatula*

	Collection	Leaf Blade	Petiole	Stem	Root	Capsule	Seed
		% Total Alkaloid Calculated to Dry Basis					
Diploid *D. stramonium*	1	0.424	0.645	0.151	0.283	0.245	0.312
	2	0.392	0.735	0.121	0.251	0.241	
	3	0.369	0.547	0.110	0.245	0.254	
Tetraploid *D. stramonium*	1	0.496	0.654	0.224	0.304	0.310	0.473
	2	0.428	0.878	0.157	0.205	0.285	
	3	0.457	0.783	0.147	0.248	0.304	
Diploid *D. tatula*	1	0.561	0.885	0.153	0.289	0.348	0.331
	2	0.439	0.824	0.093	0.332	0.291	
	3	0.456	0.677	0.113	0.309	0.326	
Tetraploid *D. tatula*	1	0.660	0.761	0.310	0.372	0.368	0.513
	2	0.511	0.758	0.251	0.255	0.335	
	3	0.634	0.599	0.171	0.339	0.357	
		% Increase in Alkaloid in Tetraploid					
D. stramonium	1	17.0	1.4	48.3	7.4	26.5	51.6
	2	9.2	19.5	29.8	−18.3	18.3	
	3	23.8	43.1	33.6	1.2	19.7	
Average		16.7	21.3	37.2	− 3.2	21.5	51.6
D. Tatula	1	17.6	−14.0	102.6	28.7	5.7	55.0
	2	16.4	− 8.0	170.0	−23.2	15.1	
	3	39.0	−11.5	51.3	9.7	9.5	
Average		24.3	−11.2	108.0	5.1	10.1	55.0

ficial assay. This procedure gave consistent results and a complete extraction of the alkaloids with a minimum of difficulty.

The results of these analyses are given in Table IV. The values shown represent the average of several individual determinations.

In addition to the previous samples, leaves with a small amount of accompanying petiole were collected from plants of diploid *D. stramonium* and diploid and tetraploid *D. tatula* grown a year earlier in San Francisco. These plants likewise were grown in rows side by side under as nearly identical conditions as possible and the leaves collected during the flowering period and air dried. Since these samples would furnish an additional comparison of the alkaloid content of these three kinds, they were also analyzed and the results are presented in Table V.

TABLE V.—TOTAL ALKALOID CONTENT OF THE LEAVES COLLECTED IN 1942

	% Total Alkaloid Calc. to Dry Basis	% Increase in Tetraploid
Diploid *D. stramonium*	0.442	....
Diploid *D. tatula*	0.416	....
Tetraploid *D. tatula*	0.539	29.6

These analyses showed a similar increase in the alkaloid content of the leaves of *D. tatula* accompanying tetraploidy but the alkaloid content of diploid *D. stramonium* was slightly higher than that of diploid *D. tatula* which was in contrast to the previous results.

TABLE VI.—COMPOSITION OF THE ALKALOID MIXTURE IN THE LEAVES

	Diploid *D. stramonium*	Tetraploid *D. stramonium*	Diploid *D. tatula*	Tetraploid *D. tatula*
% Total alkaloid calcd. to dry basis	0.328	0.398	0.417	0.466
Composition of Mixture				
% Hyoscyamine	93.5	95.0	88.0	91.4
% Atropine	1.0	0.0	3.2	1.8
% Scopolamine	5.5	5.0	8.8	6.8

Analysis of the Alkaloid Mixture.—Since tetraploidy is accompanied by various metabolic and morphologic alterations it is possible that the nature of the alkaloid mixture in *Stramonium* tetraploids would be changed. To determine whether or not such was the case, the individual alkaloids were determined in the leaves using Kuhn and Schäfer's method (11) with slight modifications. The leaves were selected for this study because they constitute a large bulk of the plant, they are the part used in medicine and recognized by the Pharmacopœia, and they contain a relatively high percentage of alkaloid. If no changes were apparent in the character of the alkaloid of the leaves it is quite unlikely that there would be any in other parts of the plant.

Because of the limited accuracy of the polariscope readings the values for atropine were but approximations and served only to indicate that little or none was present. The results of these analyses appear in Table VI.

DISCUSSION

The use of balanced tetraploids originating from the same parent seed used as a source of diploids, confines the effect on moisture, ash, and alkaloid content solely to that of tetraploidy. While the particular strains used were not identified as to

their exact type and genic constitution, they were identical in both diploid and tetraploid. The chromosome counts on both diploids and tetraploids eliminated the possible inclusion of unbalanced mutants with an excess or deficiency of chromosomes.

The leaves of *D. stramonium* grown in 1942 contained a slightly higher alkaloid content than those of *D. tatula* while those grown in 1943 were appreciably lower. The two yearly crops were grown in different localities with quite diverse climates and this variation may indicate that the two were not equally affected by the same change in environmental conditions.

The tetraploids of both varieties were considerably richer in alkaloids in most plant parts, including those which compose the bulk of the plant. Both increases and decreases were noted in root and petiole samples. The lowered content of the tetraploid *D. tatula* petioles may be due to an increase of structural elements, low in alkaloids, required to support the large leaves. The leaves of *D. tatula* were larger than those of *D. stramonium* in both diploids and tetraploids.

Although the leaves of the tetraploids were about twice the size of the corresponding diploids they possessed only about half as many leaves per plant. Flowers, seeds, and pollen of the tetraploids were also larger but there was little difference in over-all size of the plants.

The character of the individual alkaloids composing the total alkaloid mixture of the leaves was not significantly altered by tetraploidy. In all cases the mixture consisted chiefly of hyoscyamine with a small amount of scopolamine and little or no atropine.

The moisture content of the fresh leaf blades and petioles was slightly higher in the tetraploids but not sufficient to appreciably affect the yield of dry material. The petioles compose a considerable fraction of the whole leaf and add significantly to the total alkaloids because of their high content.

The total ash and acid-insoluble ash content of the leaves, petioles, and stems were also not appreciably affected. In all samples the acid-insoluble ash was far under the U. S. P. XII limit.

The Kuhn and Schäfer method gave lower total alkaloid values than the U. S. P. XII assay but showed the same increased alkaloid content of the tetraploids.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

4,4′-Di-(dimethylamino)-benzophenone
4-Dimethylaminobenzophenone
Naphthostyrile
2-Mercaptohistidine
Thiocholesterol
Androsterone
4-Amino pyridine
1-Nitro-8-naphthalene sulfonic acid
Benzanthrone
Hirudin
Strychnidine
Aspergillic acid
Pyocyanase
Eleostearic acid
Kynurenic acid
Xanthurenic acid
N,N-Dimethyl or diethyl-3,4-dihydroxy phenylalanine
N,N-Dimethyl or diethyl tyrosine
β-Dimethylaminoethyl-*p*-butylamino benzoate
4-Chloro-2,6-dimethylphenol
Calcium ricinoleate diiodide
Sodium pentathionate

The Analgesic Properties of Some 1-Acyl-3-Aminomethylureas*

By M. A. SPIELMAN† and R. K. RICHARDS†

A series of 1-acyl-3-aminomethylureas has been synthesized by the condensation of acylureas with a secondary amine and formaldehyde. Only 1-acetyl-3-piperidinomethylurea hydrochloride has a satisfactory analgesic effect. Clinical trial, however, brought out undesirable side effects. The drug is unstable in solution, and piperidine, the chief breakdown product, seems to be responsible for many of the pharmacological phenomena elicited.

EINHORN (1) found that 1-acylureas undergo a Mannich type of reaction with formaldehyde and secondary amines to give 1-acyl-3-dialkylaminomethylureas.

$$RCONHCONH_2 + CH_2O + R'_2NH \rightarrow RCONHCONHCH_2NR'_2$$

The hydrochlorides of these bases are freely soluble in water and certain members of the series, e.g., 1-isovaleryl-3-diethylaminomethylurea, are known to possess hypnotic properties (2).

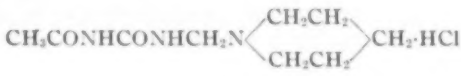
During a search for new analgesic agents, the Einhorn compounds came under consideration, and it seemed probable that if the acyl part were properly chosen the undesirable hypnotic effect might disappear and allow an analgesic effect to emerge. The diethylacetyl- and isovalerylureas prepared by Einhorn are predictably hypnotic, and the use of smaller acyls should lessen this disadvantage. Accordingly, some of the lower acylated ureas were condensed with formaldehyde and secondary amines to form the products shown in Table I.

Synthesis of the compounds gave little difficulty after a technique was evolved. Most of the trouble came from the fact that the free bases hydrolyze with extreme ease and in most instances could not be isolated in pure form. Even the hydrochlorides are not completely stable in aqueous solution. The hydrolysis products are the acylurea formed by the reversal of the condensation reaction, along with the usual secondary amine-formaldehyde condensates.

$$AcNHCONHCH_2NR_2 + H_2O \rightleftarrows AcNHCONH_2 + R_2NH + CH_2O$$
$$R_2NH + CH_2O \rightleftarrows R_2NCH_2OH \rightleftarrows R_2NCH_2NR_2$$

1 - Acetyl -3 - piperidinomethylurea hydrochloride begins to precipitate acetylurea after standing several days in aqueous solution at room temperature. The process is, of course, speeded by warming. The effect of this behavior upon pharmacological properties is discussed below.

1-Acetyl-3-piperidinomethylurea hydrochloride (compound I) proved to be the most interesting as an analgesic agent. It was given the designation AN-64 and hereafter is so called.



I

EXPERIMENTAL

The 1-acyl-3-aminomethyleneurea hydrochlorides were all prepared by refluxing the appropriate acylurea, formaldehyde or paraformaldehyde, and the secondary amine in alcoholic solution for eighteen hours. Two methods of isolation were used. In method A the crude base was separated and then converted to the hydrochloride. In method B the hydrochloride was formed in the original alcoholic solution. In both methods the yields were usually about 30–50% of theory. By the use of 1-acetyl-3-piperidinomethylurea as an example, the two methods are illustrated as follows.

Method A.—To 75 cc. of absolute alcohol was added 20 Gm. acetylurea, 17 Gm. of piperidine, and 16 cc. of formalin. The mixture was refluxed overnight, then taken to a syrup under diminished pressure. The syrup dissolved readily in 25 cc. of water, and on cooling and scratching 16 Gm. of the free base, 1-acetyl-3-piperidinomethylurea, came down as needles; m. p. 122–123°. The hydrochloride was prepared by dissolving the base in acetone and precipitating with hydrogen chloride. It crystallized well from methanol.

Method B.—A mixture of 10.2 Gm. of acetylurea, 8.5 Gm. of piperidine, 3.5 Gm. of paraformaldehyde,

* Received Aug. 31, 1945, from Abbott Laboratories, North Chicago, Ill.
† The authors are indebted to E. F. Shelberg and L. F. Reed for all microanalyses and to K. K. Kueter and W. B. Brownell for valuable technical assistance.

and 50 cc. of absolute alcohol was refluxed overnight, cooled, and filtered. The precipitate was washed with 50 cc. more of alcohol. The filtrate and washings were acidified with alcoholic HCl. Fourteen grams of crystalline hydrochloride soon came down; m. p. 194–196°.

Table I lists the compounds prepared with the method of preparation, melting points, and analytical figures.

TABLE I.—1-ACYL-3-AMINOMETHYLUREA HYDROCHLORIDES

Compound No.	Acyl	Amine	Method	M. P. ° C.	Formula	N, % Calcd.	N, % Found
I	Acetyl	Piperidine	A, B	195–196	$C_8H_{18}N_3O_2Cl$	17.8	17.7
II	Carbethoxy	Piperidine	A	110–112	$C_{10}H_{20}N_3O_3Cl$	15.8	15.6
III	Acetyl	Diethylamine	A	155–157	$C_8H_{18}N_3O_2Cl$	18.8	19.1
IV	Acetyl	γ-Pipecoline	A	180	$C_{10}H_{20}N_3O_2Cl$	16.7	16.4
V	Acetyl	Morpholine	A, B	185–186	$C_8H_{16}N_3O_3Cl$	17.6	17.4
VI	Propionyl	Piperidine	A	168–170	$C_{10}H_{20}N_3O_2Cl$	16.7	16.8
VII	Butyryl	Piperidine	A	151–154	$C_{11}H_{22}N_3O_2Cl$	15.9	15.8

TABLE II.—THRESHOLD OF TWITCH RESPONSE

Dog	Drug	Dose, Mg./Kg.	Threshold (Watts) after Time Elapsed 0 Min.	45 Min.	90 Min.	180 Min.	Increase, %	Remarks
1	AN-64	150, subcu.	280	330	330	340	21.3	
2	AN-64	150, orally	270	270	290	280	7.4	Vomited
3	AN-64	150, orally	285	310	320	320	12.3	Vomited
4	AN-64	250, subcu.	240	300	320	300	33.3	
5	Aspirin	100, orally	310	330	330	330	9.7	
6	Aspirin	100, orally	200	260	260	250	30.0	Vomited
7	Aspirin	150, orally	270	280	290	300	11.1	

The following were isolated as free bases in a state pure enough for reasonably good analytical values:

Anal.—Calcd. for 1-acetyl-3-piperidinomethylurea, $C_9H_{17}N_3O_2$: N, 21.1. Found: N, 21.1. M. p. 123–124°.

Anal.—Calcd. for 1-acetyl-3-morpholinomethylurea, $C_8H_{15}N_3O_3$: N, 20.9. Found: N, 20.4. M. p. 150°.

This is undoubtedly less pure than the specimen reported by Weaver, *et al.*, to melt at 161° (3).

Anal.—Calcd. for 1-propionyl-3-piperidinomethylurea, $C_{10}H_{19}N_3O_2$: N, 19.7. Found: N, 20.0. M. p. 97–98°.

PHARMACOLOGY

It may be stated at the outset that of the present series of compounds only one, 1-acetyl-3-piperidinomethylurea hydrochloride (AN-64) showed any interesting pharmacological properties. Toxicity studies, for reasons to follow, will be discussed later.

We have studied AN-64 with particular attention to possible analgesic action. Tests for analgesia in rats were conducted by the method of Macht and Maeht (4) and D'Amour and Smith (5). In the mouse, the technique of Kueter and Richards (6) was used. The results, after taking into account the variability of animal response to any of the methods, were indicative of a moderate analgesic potency.

Comparison with aspirin was possible only in the experiments carried out on dogs since aspirin did not produce definite analgesic effects in mice or rats with the above procedures. In dogs we used the method of Hardy, Wolff, and Goodell (7) as modified by Workman and Andrews (8). This test is based upon the occurrence of a twitch reflex of the platysma muscle of the dog when the rays of a light bulb are focused on the skin by means of a lens system. The watt output of the bulb is regulated by a Variac transformer, and the effect of an analgesic drug is measured by the increase in wattage necessary to evoke the twitch response after administration of the drug. Table II shows comparative experiments on dogs receiving AN-64 by mouth or by injection and others given aspirin. The results indicate that AN-64 tends to produce nausea if given orally but that it compares favorably with aspirin if injected subcutaneously. By the latter route it does not produce noticeable side effects.

Clinical trial sustained the animal work. Analgesia in humans was pronounced when AN-64 was administered orally or parenterally, but a high incidence of side effects made it improbable that the compound could ever have extensive medicinal use. The untoward effects took the form of nausea, faintness, flushing, and in one case, transitory collapse.

The study of the circulatory effects of AN-64 in dogs under Nembutal anesthesia made possible some interesting observations. If 10–15 mg./Kg. AN-64 were injected intravenously as a freshly prepared solution, only a slight and evanescent drop in blood pressure was observed, and practically no effect on respiration could be noted. However, when the same solution was injected after standing at room temperature for a few days, a marked pressor effect appeared. As expected, the change was accelerated by simply boiling the solution.

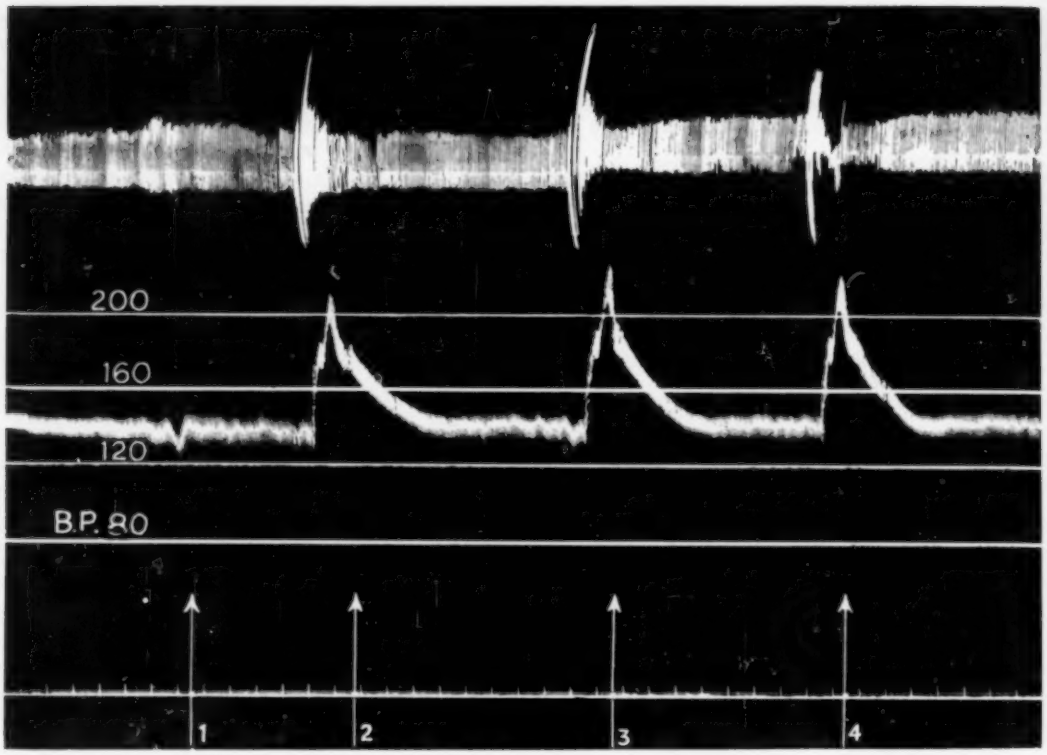


Fig. 1.—Dog, Nembutal anesthesia. Upper tracing respiration, lower tracing blood pressure, Time signals 1 min.
1. Sol'n A. Fresh AN-64, 10 mg./kg.
2. Sol'n B. Boiled AN-64, equivalent to the piperidine content of 2 mg./kg. AN-64.
3. Sol'n C. Dipiperidine methane, equivalent to the piperidine content of 2 mg./kg. AN-64.
4. Sol'n D. Piperidine hydrochloride, equivalent to the piperidine content of 2 mg./kg. AN-64.

The structural formula of AN-64 does not immediately confer the impression that the piperidine constituent of the compound would exhibit the typical action of this alkaloid. Such, however, is the case if decomposition takes place, and from the type of action exerted, it is plain that piperidine is split off in the aging or heating of an aqueous solution. This is in keeping with the purely chemical observation that AN-64 is a substance of very limited stability.

The correctness of the interpretation was established by the following experiment. A fresh 5% solution of AN-64 was prepared (solution *A*). A part of it was boiled for ten minutes after which the evaporated water was replaced (solution *B*). Solution *C* was made up of dipiperidinomethane hydrochloride in a concentration having the exact piperidine equivalence of solution *A* or *B* if they were to yield their piperidine quantitatively on decomposition. Solution *D* contained piperidine hydrochloride as such, also in equivalence to solution *A*. The significance of the experiment may be made clear by reference to the chemical part of this paper, for, as shown in Equations 1 and 2, each molecule of AN-64 contains, potentially, about 50% of piperidine hydrochloride or the related products of condensation with formaldehyde. As the tracing shows, the injection of freshly prepared AN-64 solution was practically without effect, while a decided pressor action and a stimulation of respiration was evoked by a boiled solution of that drug. It is striking that qualitatively and quantitatively almost congruent responses followed the administration of doses of piperidine or dipiperidinomethane in amounts equal to the piperidine content theoretically obtained from the decomposition of AN-64.

Dixon (9), who has discussed the action of piperidine, draws attention to the fact that the alkaloid shows great similarity to nicotine and coniine. Its action, however, is milder. The pressor effect is predominantly due to peripheral vasoconstriction and to the effect upon the autonomic ganglia. We have noticed a slight increase in pulse rate, but we have made no attempt to analyze the purport of this in detail. The experiment illustrated in Fig. 1, along with other observations, showed that there was little tendency to tachyphylaxis if moderate doses of piperidine were given. The immediate respiratory response was very pronounced, and consisted of an increase of rate as well as depth of respiration. However, this phenomenon was more short-lived than was the pressor action. Of interest is the characteristic and consistent shape of the pressor response curve with its slight but distinctive step

at about two-thirds of the maximum height of the ascending limb. It should be noted that no cardiac arrhythmia appeared in this experiment. In others they were observed if excessive blood pressure responses were elicited.

Toxicity studies, conducted by intraperitoneal injection in mice, are summarized on Table III. The first vertical column gives results obtained with a freshly prepared solution of AN-64; the second one, those with an identical solution after boiling. This latter procedure led to a moderate increase in toxicity. It will be noted that the toxicity of piperidine, shown in the third column, is somewhat more than twice that of the boiled AN-64. As mentioned above, piperidine constitutes about one-half of the AN-64 molecule. Further experiments showed that the toxicity of a dipiperidinomethane solution is also parallel to its piperidine content. The symptoms brought about in mice with toxic doses of any of the four solutions were identical. They consisted of depression, labored respiration, followed frequently but not regularly by convulsive seizures which were always fatal. The heart beat outlasted respiration. The animals showed the first symptoms 5 to 10 minutes after injection and all fatalities occurred within $^1/_2$ hour. While no definite difference in the time necessary to produce toxic response and death was evident among the four solutions, the splitting off of the piperidine component from the AN-64 molecule does not appear to be an immediate reaction. This can be concluded from the lack of circulatory and respiratory effect even if relatively large doses of the drug are introduced intravenously, and also from the lower toxicity of the unhydrolyzed molecule.

Daily administration of 80 mg./Kg. by intraperitoneal injection to young rats for twenty-one days did not depress their growth nor exert significant toxic effect upon the hematopoietic system. There were some fatty changes in the livers of a few animals, but no other pathologic findings.

Since the analgesia observed in the animals persisted sometimes for a considerable period, that is, two to three hours, it must be left undecided as to the form in which the drug exerted this specific action. Piperidine itself seems not to be known to possess analgesic properties in animals or in man. In this connection the recent note of Euler (10) is of interest, indicating that piperidine in one form or another is a normal constituent of urine. It should also be pointed out that substances such as compounds II, VI, and VII, which possess the piperidine moiety, but not the acetylurea part of the molecule, are devoid of analgesic effects.

1-Acetyl-3-diethylaminomethylurea hydrochloride was also strongly analgesic but in a very erratic manner, due, probably, to intrinsic instability, and was therefore not studied extensively. The other members of the series showed no interesting properties.

TABLE III.—COMPARATIVE TOXICITY OF AN-64 AND PIPERIDINE

Intraperitoneal Injection in Mice (No. died/No. Injected)

Dose, Mg./Kg.	AN-64, Fresh Soln.	AN-64, Boiled Soln.	Piperidine HCl
200	.....	.....	0/2
250	.....	0/8	.....
300	.....	.....	3/11
400	.....	.....	13/14
500	0/14	1/8	5/6
700	.....	.....	.....
800	5/18	5/8	.....
900	.....	.....	.....
1000	15/19	8/8	.....
Approximate LD_{50}	890 mg./Kg.	730 mg./Kg.	370 mg./Kg.

SUMMARY

A series of 1-acyl-3-aminomethylureas has been synthetized. Both the acyl and the amino groups have been varied. All were tested for analgesic effect, but only one, 1-acetyl-3-piperidinomethylurea, showed any attributes of pharmacological interest. It possesses low toxicity and has definite analgesic properties, but side effects render it unfit for clinical use. It is unstable in solution, and its breakdown to give piperidine explains in part some of its pharmacological effects.

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Book Reviews

Physical Methods of Organic Chemistry. Volume I. Edited by ARNOLD WEISSBERGER. Science Publishers, Inc., New York, 1945. vii + 736 pp. 23 x 14.5 cm. Price, $8.50.

As physical-chemical methods improved, largely due to better instrumentation, their use in organic chemistry increased. A number of specialists in interpreting organic chemistry in physical terms, naturally appeared on the scene. This gradual change in outlook necessitated the publishing of monographs in this hybrid field. *Physical Methods of Organic Chemistry* was written, collaboratively, to fulfill such a need.

Volume I of this reference contains chapters on: Determination of Melting and Freezing Temperatures; Determination of Boiling and Condensation Temperatures; Determination of Density; Determination of Solubility; Determination of Viscosity; Determination of Surface and Interfacial Tension; Parachor; Determination of Properties of Monolayers and Duplex Films; Determination of Osmotic Pressure; Determination of Diffusivity; Calorimetry; Microscopy; Determination of Crystal Form; Crystallochemical Analysis; X-Ray Diffraction; Electron Diffraction; and Refractometry. It is understood that this volume is to be followed shortly by Volume II, containing chapters on: Spectroscopy and Spectrophotometry; Colorimetry, Photometric Analysis, and Fluorimetry; Polarimetry; Determination of Dipole Moments; Conductometry; Potentiometry; Polarography; Determination of Magnetic Susceptibility; Determination of Radioactivity; and Mass Spectrometry

Since individual chapters are written by different authors a certain degree of unevenness would be anticipated, but good editorship has maintained a surprising balance of quality. In some cases, however, the balance between the practical and the theoretical is not as good as it might be.

Certain chapters will be of limited interest due to lack of equipment. For example, many readers will be less interested in the section on "X-Ray Diffraction" because of the unavailability of the necessary equipment. On the other hand, all organic chemists can read with profit such sections as that on "Determination of Melting and Freezing Temperatures." This chapter opens with a section on the theoretical discussion of ideal systems as well as those which deviate from ideality. In this section is one of the finest explanations of cooling curves, in terms of the phase rule, that the reviewer has ever been privileged to read. The practical discussion of apparatus to be used for such methods is also helpful. After a section on thermometers, in which partial immersion stem corrections are discussed, the author develops the theory and practice of molecular-weight determination by freezing methods.

Pharmaceutical chemists will be especially interested in a chapter on density. The effect of temperature and impurities on density, as well as finer points in 5th place pycnometry, are discussed adequately for the ordinary worker. Dilatometric as well as buoyancy methods are covered also.

The reviewer is so enthusiastic about this convenient reference book that the appearance of Volume II is anticipated with equal pleasure. Unfortunately, the first volume is not indexed, but it is understood that a very complete index, covering both volumes, will appear in the second volume. If the index is as complete as promised, the usefulness of both volumes will be greatly enhanced.—MELVIN W. GREEN

Major Instruments of Science and Their Applications to Chemistry. Edited by R. E. BURK and OLIVER GRUMMITT. Interscience Publishing Company, New York, 1945. 151 pp. 15 x 23 cm. Price $3.50.

The instruments described in this book are: electron diffraction apparatus, the electron microscope, X-ray apparatus, the spectroscope, and the infrared spectrometer.

This book is in reality Volume IV of a series known as "Frontiers in Chemistry," a series of lectures given to the graduate students at Western Reserve University. Each chapter is based upon a lecture by an authority in each field.

Each author has attempted to give the highpoints of his particular field of specialization. The basic construction of the instrument is given and the theory upon which it operates. This procedure is followed by some examples of its application.

Since these tools are being used more and more in the physical sciences, many persons in the various branches of pharmaceutics will be interested in this introductory book. —MELVIN W. GREEN.